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(71) Applicant *(for all designated States except US)*: **CHIRON CORPORATION** [US/US]; 4560 Horton Street, Emeryville, CA 94608 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **POLO, John, M.** [US/US]; 221 Witham Road, Encinitas, CA 92024 (US). **DUBENSKY, Thomas, W., Jr.** [US/US]; 6 Pacific Avenue, Piedmont, CA 94611 (US). **FROLOV, Ilya** [US/US]; 200 Tanglewood Drive, St. Louis, MO 63124 (US). **GARDNER, Jason, P.** [US/US]; 4560 Horton Street, Emeryville, CA 94608 (US). **OTTEN, Gillis** [US/US]; 35 Williams Lane, Foster City, CA 94404 (US). **BARNETT, Susan** [US/US]; 4240 21st Street, San Francisco, CA 94114 (US). **DRIVER, David, A.** [US/US]; 1015 Santa Estrella, Solana Beach, CA 92075 (US).

(74) Agents: **BLACKBURN, Robert, P.**; Chiron Corporation, 4560 Horton Street, Emeryville, CA 94608-2916 et al. (US).

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(54) Title: COMPOSITIONS AND METHODS FOR GENERATING AN IMMUNE RESPONSE UTILIZING ALPHAVIRUS-BASED VECTOR SYSTEMS

(57) Abstract: Methods are provided for generating immune responses utilizing alphavirus-based vector systems. Specifically, compositions and methods for priming the immune system of an animal using a first immunogenic composition followed by boosting the immune system with a second immunogenic composition are disclosed.

COMPOSITIONS AND METHODS FOR GENERATING AN IMMUNE RESPONSE UTILIZING ALPHAVIRUS-BASED VECTOR SYSTEMS

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application Nos. 60/191,363, filed March 22, 2000, which application is incorporated by reference in its entirety.

Background of the Invention

The present invention relates generally to gene-based vaccines and therapeutics; and more specifically, to compositions and methods that increase the efficiency of alphavirus-based vector systems used for such vaccines and therapeutics.

Alphaviruses comprise a group of genetically, structurally, and serologically related arthropod-borne viruses of the *Togaviridae* family. These viruses are distributed worldwide, and persist in nature through a mosquito to vertebrate cycle. Birds, rodents, horses, primates, and humans are among the identified alphavirus vertebrate reservoir/hosts.

Twenty-six known viruses and virus subtypes have been classified within the alphavirus genus utilizing the hemagglutination inhibition (HI) assay. This assay segregates the 26 alphaviruses into three major complexes: the Venezuelan equine encephalitis (VEE) complex, the Semliki Forest (SF) complex, and the western equine encephalitis (WEE) complex. In addition, four other viruses, eastern equine encephalitis (EEE), Barmah Forest, Middelburg, and Ndumu, receive individual classification based on the HI serological assay.

Members of the alphavirus genus also are further classified into one of two groups, according to the clinical symptoms they exhibit as a result of infection in humans. The first group is alphaviruses associated primarily with encephalitis, and the second group is alphaviruses associated primarily with fever, rash, and polyarthritis. Included in the first group are the VEE and WEE complexes, and EEE. In general, infection with this group can result in permanent sequelae, including death. In the second group is the SF complex, comprised of the individual alphaviruses Semliki Forest,

Sindbis, Ross River, Chikungunya, O'nyong-nyong, and Mayaro. Although serious epidemics have been reported, infection by viruses of this group is generally self-limiting, without permanent sequelae.

Sindbis virus is the prototype member of the *Alphavirus* genus of the *Togaviridae* family. Its replication strategy is well characterized and serves as a model for other alphaviruses (Strauss and Strauss, *Microbio. Rev. 58*:491-562, 1994). The genome of Sindbis virus (like other alphaviruses) is an approximately 12 kb single-stranded, positive-sense RNA molecule that is capped and polyadenylated. Genome RNA is contained within a virus-encoded capsid protein shell which is, in turn, surrounded by a host-derived lipid envelope from which two viral-specific glycoproteins, E1 and E2, protrude as spikes from the virion surface. Certain alphaviruses (*e.g.*, SF) also maintain an additional protein, E3, which is a cleavage product from the E2 precursor protein, PE2.

After virus particle absorption to target cells, penetration, and uncoating of the nucleocapsid to release viral genomic RNA into the cytoplasm, the replication process is initiated by translation of four nonstructural replicase proteins (nsP1-nsP4) from the 5' two-thirds of the viral genome. The four nsPs are translated as one of two polyproteins (nsP123 or nsP1234), and processed post-translationally into mature monomeric proteins by an active protease in the C-terminal domain of nsP2. Both of the nonstructural polyproteins and their derived monomeric units may participate in the RNA replication process, which involves nsP binding to the conserved nucleotide sequence elements (CSEs) present at the 5' and 3' ends, and an internal subgenomic junction region promoter.

The positive strand genome RNA serves as template for the nsP-catalyzed synthesis of a full-length complementary negative strand RNA. Synthesis of the negative strand RNA is catalyzed by binding of a nsP complex to the 3' terminal CSE of the positive strand genome RNA. The negative strand, in turn, serves as template for the synthesis of additional positive strand genome RNA, as well as an abundant subgenomic RNA, initiated internally at the junction region promoter. Synthesis of additional positive strand genome RNA occurs after binding of a nsP complex to the 3'

terminal CSE of the complementary negative strand genome-length RNA template. Synthesis of the subgenomic mRNA from the negative-strand RNA template is initiated from the junction region promoter. Thus, the 5' end and junction region CSEs of the positive strand genome RNA are functional only after being transcribed into the negative strand RNA complement (*i.e.*, the 5' end CSE is functional when it is the 3' end of the genomic negative stranded complement).

Alphavirus structural proteins (sPs) are translated from the subgenomic RNA, which represents the 3' one-third of the genome, and like the nsPs, are processed post-translationally into the individual proteins (see Figure 1). Translation of this subgenomic mRNA produces a single polyprotein consisting of the structural proteins capsid (C) glycoprotein E2 and glycoprotein E1, plus the corresponding leader/signal sequences (E3, 6k) for glycoprotein insertion into the endoplasmic reticulum. The structural gene polyprotein is processed into the mature protein species by a combination of viral (capsid autoprotease) and cellular proteases (*e.g.*, signal peptidase). Alphavirus structural proteins are produced at very high levels due to the abundance of subgenomic mRNA transcribed, as well as the presence of a translational enhancer element (Frolov and Schlesinger, *J. Virol. 68*:8111-8117, 1994; Sjoberg et al., *Bio/Technol. 12*:1127-1131, 1994) within the mRNA, located in the capsid gene coding sequence. Because all structural proteins are synthesized at equimolar ratios, as part of the polyprotein, the translation enhancer element exerts its effect equally on each of the genes.

Several members of the Alphavirus genus are being developed as expression vectors, including, for example, Sindbis virus (Xiong et al., *Science 243*:1188-1191, 1989; Hahn et al., *Proc. Natl. Acad. Sci. USA 89*:2679-2683, 1992; Dubensky et al., *J. Virol. 70*:508-519, 1996), Semliki Forest virus (Liljestrom, *Bio/Technology 9*:1356-1361, 1991), and Venezuelan equine encephalitis virus (Pushko et al., *Virology 239*:389-401, 1997). The general strategy for construction of alphavirus-based expression vectors has been to substitute the viral structural protein genes with a heterologous gene, maintaining transcriptional control via the highly active subgenomic RNA promoter. RNA vectors having this configuration are self-amplifying, and thus are termed RNA "replicons". The replicons may be synthesized *in vitro* from cDNA using

a bacteriophage promoter (Xiong et al., *ibid*; Liljestrom et al., *ibid*; Pushko et al., *ibid*), or generated *in vivo* directly from DNA when linked to a eukaryotic promoter (Dubensky et al., *ibid*; US Patents 5,814,482 and 6015686; WO 97/38087). Because the vector replicons do not express the alphavirus structural proteins necessary for packaging into recombinant alphavirus particles, these proteins must be provided in *trans*. One alphavirus, Venezuelan equine encephalitis virus, and its derived recombinant vector particles have been shown to be lymphotropic and infect murine dendritic cells (Caley et al., *J. Virol. 71*:3031-3038, 1997; MacDonald et al., *J Virol. 74*:914-22, 2000). However, no alphavirus or alphavirus variant was demonstrated to infect human dendritic cells, macrophages or antigen presenting cells.

The present invention discloses novel compositions and methods for generating an enhanced immune response utilizing alphavirus-based vector systems, and further, provides other related advantages.

Brief Summary Of The Invention

Briefly stated, the present invention provides compositions and methods for generating an enhanced immune response utilizing alphavirus-based vector systems. Within one aspect of the present invention, isolated alphaviruses and recombinant alphavirus particles are provided which infect human dendritic cells, (with the proviso that the alphavirus is not ATCC # VR-2526 or said alphavirus particle is not generated in total from ATCC # VR-2526). Within another aspect, isolated alphaviruses and recombinant alphavirus particles are provided which infect non-human dendritic cells (with the proviso that the alphavirus is not a Venezuelan equine encephalitis virus or ATCC # VR-2526, or, that the recombinant alphavirus particle is not derived in total from Venezuelan equine encephalitis virus or ATCC # VR-2526). Within yet another aspect, isolated alphaviruses and recombinant alphavirus particles are provided which infect human macrophages. Within related aspects, isolated alphaviruses and recombinant alphavirus particles are provided which infect human antigen presenting cells, (with the proviso that the alphavirus is not ATCC # VR-2526 or said alphavirus particle is not generated in total from ATCC # VR-2526).

Within certain embodiments of the above, the alphavirus or recombinant alphavirus particle has an amino acid substitution in the E2 glycoprotein as compared to wild-type, for example, at residue 158, 159, 160, 161, or, 162. Within preferred embodiments, the amino acid substitution is at E2 residue 160. Within other embodiments, the alphavirus has an amino acid deletion or insertion in the E2 glycoprotein. Within further embodiments, the alphavirus is a Semliki Forest virus, a Ross River virus, a Venezuelan equine encephalitis virus, a Sindbis virus, or ATCC No. VR-2643. Also provided are nucleic acid molecules which encode the above described alphaviruses (such as, for example, as provided in Figures 2B and 2C).

Within other aspects of the present invention alphavirus structural protein expression cassettes are provided, comprising a promoter operably linked to a nucleic acid sequence encoding one or more alphavirus structural proteins from an alphavirus as described above. Within a related aspect, alphavirus structural protein expression cassettes are also provided comprising a promoter operably linked to a nucleic acid sequence encoding alphavirus structural proteins, wherein said nucleic acid sequence comprises a sequence encoding glycoprotein E2, and wherein said sequence encodes a mutation (*e.g.*, a substitution, deletion, or insertion, as compared to wild-type) in the E2 glycoprotein. Within various embodiments, the mutation is a substitution at E2 residue 158, 159, 160, 161, or 162. Within preferred embodiments, the mutation confers to an alphavirus or recombinant alphavirus particle packaged with said E2 glycoprotein the ability to infect human dendritic cells. Within related aspects alphavirus packaging cell lines are provided comprising a host cell and an alphavirus structural protein expression cassette as described above, as well as an alphavirus producer cell line comprising such packaging cells and a vector selected from the group consisting of an alphavirus RNA vector replicon, alphavirus vector construct, and a eukaryotic layered vector initiation system. Further, recombinant alphavirus particles also are provided which can be produced from the above packaging or producer cell lines.

Within yet other aspects of the present invention, methods are provided for introducing a selected sequence into a cell, comprising the step of infecting with a recombinant alphavirus particle or otherwise introducing into a cell an alphavirus replicon as

described above, such that the selected sequence is introduced into the cell. A wide variety of selected sequences may be introduced into a cell or cells, including for example, heterologous sequences such as those encoding proteins (*e.g.*, peptides, antigens, antibodies, cytokines, lymphokines), and non-protein encoding sequences such as ribozymes. In addition, the recombinant alphavirus particles may be designed to express one or more sequences (*e.g.*, an immune enhancer, cytokine, chemokine, for example, IL-2, IL-10, IL-12, gamma interferon, GM-CSF, MIP3□, MIP3□, molecules with related function). Further, the recombinant alphavirus particles may be administered utilizing ex vivo or in vivo techniques. Further, within certain embodiments the recombinant alphavirus particles may be utilized with a wide variety of cells, cell populations, or tissues, including for example, a population of cells containing dendritic cells. Within yet further embodiments, the recombinant alphavirus particles may be introduced as a composition, *e.g.*, with enhancers such as cytokines or chemokines (*e.g.*, IL-2, IL-10, IL-12, gamma interferon, GM-CSF), other vectors, or adjuvants.

Within yet further aspects of the invention, alphavirus vector constructs are provided comprising (a) a 5' promoter which initiates synthesis of viral RNA *in vitro* from cDNA, (b) a 5' sequence which initiates transcription of alphavirus RNA, (c) a nucleic acid molecule which operably encodes all four alphaviral nonstructural proteins, (d) an alphavirus RNA polymerase recognition sequence; and (e) a 3'polyadenylate tract, wherein the nucleic acid sequence which operably encodes all four alphaviral nonstructural proteins contains a mutation in at least one nonstructural protein selected from the group consisting of a mutation in nsP1 residues 346, 441, 473, nsP2 residues 438, 622, 634, 715, nsP3 residues, 417, 456, 505, and nsP4 residue 266.

Within yet another aspects of the present invention, eukaryotic layered vector initiation systems are provided, comprising a 5' promoter capable of initiating *in vivo* the 5' synthesis of alphavirus RNA from cDNA, a sequence which initiates transcription of alphavirus RNA following the 5' promoter, a nucleic acid molecule which operably encodes all four alphaviral nonstructural proteins, an alphavirus RNA polymerase recognition sequence, and a 3' polyadenylate tract, wherein the nucleic acid sequence which operably encodes all four alphaviral nonstructural proteins contains a mutation

in at least one nonstructural protein selected from the group consisting of a mutation in nsP1 residues 346, 441, 473, nsP2 residues 438, 622, 634, 715, nsP3 residues, 417, 456, 505, and nsP4 residue 266, as compared to wild-type.

Within further embodiments alphavirus RNA vector replicons capable of translation in a eukaryotic system are provided, comprising a 5' sequence which initiates transcription of alphavirus RNA, a nucleic acid molecule which operably encodes all four alphaviral nonstructural proteins, an alphavirus RNA polymerase recognition sequence and a 3' polyadenylate tract, wherein the nucleic acid sequence which operably encodes all four alphaviral nonstructural proteins contains a mutation in at least one nonstructural protein selected from the group consisting of a mutation in nsP1 residues 346, 441, 473, nsP2 residues 438, 622, 634, 715, nsP3 residues, 417, 456, 505, and nsP4 residue 266, as compared to wild-type.

Within another aspect of the invention, methods are provided for generating an immune response in a warm-blood animal, comprising the step of administering to a warm-blooded animal any of the recombinant alphavirus particles described above that further comprise a heterologous sequence encoding an antigen from a virus, bacteria, fungus, parasite or cancerous cell, or a heterologous sequence encoding a cytokine, lymphokine, or chemokine.

Within a further aspect of the invention, methods are providing for generating an immune response within a warm-blooded animal, comprising the step of administering to a warm blooded animal one or more selected antigens by a first method (*e.g.*, alphavirus vector particles, eukaryotic layered vector initiation system, DNA, protein, non-alphavirus viral vector), followed by administering to the same animal the same or similar antigen (*e.g.*, modified form thereof or related antigen) by a second method (*e.g.*, alphavirus vector particles, eukaryotic layered vector initiation system, DNA, protein, non-alphavirus viral vector), wherein the first and second methods are different, with the proviso that either the first method or the second method or both methods is selected from the group consisting of alphavirus vector particles, RNA vector replicons or eukaryotic layered vector initiation systems. Within various

embodiments of the invention, the immune response may be generated to treat a disease, and/or, to prevent a disease (*e.g.* as a prophylactic or therapeutic vaccine).

Within another aspect of the invention, non-lymphotropic alphaviruses are provided, wherein the alphaviruses are capable of infecting dendritic cells (DC tropic). In one embodiment the dendritic cells are immature dendritic cells (*e.g.*, CD1a+, CD86dim, $CD83^-$). In another embodiment, the alphaviruses are selected from the group consisting of Sindbis virus, Semliki Forest virus, and Ross River virus. In yet another embodiment, the alphavirus is a Sindbis virus that has an amino acid encoded at residue 160 of glycoprotein E2 that is not glutamic acid. Within a further embodiment, the alphavirus is not a Venezuelan equine encephalitis virus or Sindbis virus CMCC #4639 (deposited with the ATCC on April 2, 1996: VR-2526). Within a further embodiment the alphavirus has the nucleic acid sequence set forth in Figure 2B, or, a nucleic acid sequence which but for the redundancy of the genetic code encodes the same amino acid sequence. Within other aspects of the invention, non-lymphotropic alphaviruses are also provided, for example, as disclosed in Figure 2C, or, a nucleic acid sequence which but for the redundancy of the genetic code encodes the same amino acid sequence. Also provided are alphavirus structural protein expression cassettes, RNA vector replicons, alphavirus vector particles, and eukaryotic layered vector initiation systems which are obtained or derived from, at least in part, one or more of the above-noted alphaviruses.

Within another aspect of the invention, alphavirus vector particles are provided, wherein the alphavirus vector particles are capable of infecting culture (*e.g.*, primary) human dendritic cells with an efficiency of infection (percentage infected) greater than their efficiency for infecting cultured (*e.g.*, primary) murine dendritic cells. In one embodiment, the alphavirus vector particles can infect human dendritic cells with an efficiency at least 50%, 100%, or 200% greater than their efficiency for infecting murine dendritic cells. In another embodiment the dendritic cells are immature human dendritic cells (*e.g.*, CD1a+, CD86dim, $CD83^-$). In yet another embodiment, the alphavirus vector particles are derived from an alphavirus selected from the group consisting of a Sindbis virus, Semliki Forest virus, Ross River virus, and Venezuelan equine encephalitis virus. In yet another embodiment, the alphavirus vector particles

are from a Sindbis virus that has an amino acid encoded at residue 160 of glycoprotein E2 that is not glutamic acid. Within a further embodiment, the alphavirus vector particles are not derived from Sindbis virus CMCC #4639 (deposited with the ATCC on April 2, 1996: VR-2526). Within a further embodiment, the alphavirus vector particles are derived from the nucleic acid sequence set forth in Figure 2B, or, a nucleic acid sequence which but for the redundancy of the genetic code encodes the same amino acid sequence. Also provided are alphavirus structural protein expression cassettes, RNA vector replicons, and Eukaryotic Layered Vector Initiation Systems which are obtained or derived, at least in part, from one or more of the above-noted alphaviruses.

Within other aspects of the present invention, expression cassettes are provided comprising a promoter (*e.g.*, a bacteriophage promoter for *in vitro* use, or an RNA pol II promoter for *in vivo* use) operably linked to a full-length cDNA clone of a DC tropic alphavirus from above, such that transcription of the full-length cDNA clone yields an RNA that initiates a productive viral infection when introduced into the cytoplasm of a susceptible cell. Also provided are alphavirus vector constructs, RNA replicons, cDNA vector construct, ELVIS, and the like, which are derived from the above DC tropic alphaviruses. Within certain embodiments, vectors are provided comprising a 5'-promoter (bacteriophage or RNApolII) operably linked to the alphavirus vector cDNA sequence, subgenomic junction region promoter, heterologous gene to be expressed, and a polyadenylate tract.

A wide variety of antigens may be expressed from the alphavirus-based vector systems, including for example, antigens or peptides from a pathogenic agent (*e.g.*, a cancerous cell, virus, bacteria, fungi, or parasite).

Within other aspects of the invention, a method of introducing and expressing a heterologous sequence in human dendritic cells in vivo or in vitro (*e.g.*, for use in biological assays) is provided, comprising: infecting a population of human cells containing dendritic cells and/or dendritic cell precursors with a recombinant alphavirus vector particle according to the present invention for a time and under conditions necessary for intracellular expression of the replicon encoded heterologous

gene within the dendritic cell, said alphavirus vector particle containing the heterologous sequence, with the proviso that said alphavirus vector particle is not derived entirely from Sindbis virus CMCC #4639 (deposited with the ATCC on April 2, 1996: VR-2526). However, such particle may contain a portion of CMCC #4639. Within certain embodiments, the alphavirus vector particle is from at least a portion of a Sindbis virus, Semliki Forest virus, Venezuelan equine encephalitis virus or Ross River virus.

Within another aspect of the invention, a method of introducing and expressing a heterologous sequence in human dendritic cells and also inducing activation and/or maturation of said dendritic cells is provided, comprising: infecting a population of human cells containing dendritic cells and/or dendritic cell precursors with a recombinant alphavirus vector particle according to the present invention for a time and under conditions necessary for intracellular expression of the replicon encoded heterologous gene and activation and/or maturation of the dendritic cells, said alphavirus vector particle containing the heterologous sequence. Also provided are alphavirus vector particles comprising a vector replicon (as described in more detail below) and structural proteins necessary for particle formation, with the proviso that at least one structural protein of the vector particle is from a DC tropic alphavirus and said alphavirus vector particle is not derived entirely from Sindbis virus CMCC #4639 (deposited with the ATCC on April 2, 1996: VR-2526; however, such particles may contain a portion of CMCC #4639). Within certain preferred embodiments, the vector replicon further comprises a gene encoding an antigen from a pathogenic agent.

Within other aspects of the invention, a method of introducing and expressing a heterologous sequence in dendritic cells is provided, comprising the step of infecting a population of cells containing dendritic cells and/or dendritic cell precursors with a recombinant alphavirus vector particle, the alphavirus vector particle containing the heterologous sequence and with the proviso that the alphavirus vector particle is not derived entirely from Venezuelan equine encephalitis virus or Sindbis virus CMCC #4639 (deposited with the ATCC on April 2, 1996: VR-2526 (however, such particle may contain a portion of VEE or CMCC #4639)). Within certain embodiments, the

alphavirus vector particle is from at least a portion of a Sindbis virus, Semliki Forest virus, or Ross River virus.

In further embodiments of the invention, the dendritic cells are immature dendritic cells. In another embodiment of the invention, said method of introducing and expressing a heterologous sequence in dendritic cells is carried out *in vitro*. Such dendritic cells may be removed from peripheral blood by apheresis or other means, and/or separated or enriched from bone marrow, or from cultured and/or expanded or differentiated hematopoetic cells (see, *e.g.*, U.S. Patent Nos. 4,927,750, 5,643,786, 5,646,004, 5,648,219, 5,648,248, 5,663,051, 5,788,963, 5,811,297, 5,851,756, 5,866,115 and 5,871,728; see also the Examples). In yet another embodiment of the invention, the method of introducing and expressing a heterologous sequence in dendritic cells is carried out *in vivo*, in a warm-blooded animal (*e.g.*, by subcutaneous, intradermal, intramuscular, intranasal, intravenous injection).

Within other aspects of the invention, a method of introducing and expressing a heterologous sequence in human macrophages or antigen presenting cells in vivo or in vitro (*e.g.*, for use in biological assays) is provided, comprising: infecting a population of cells containing human macrophages or antigen presenting cells with a recombinant alphavirus vector particle according to the present invention for a time and under conditions necessary for intracellular expression of the replicon encoded heterologous gene within the dendritic cell, said alphavirus vector particle containing the heterologous sequence, with the proviso that said alphavirus vector particle is not derived entirely from Sindbis virus CMCC #4639 (deposited with the ATCC on April 2, 1996: VR-2526 (however, such particle may contain a portion of CMCC #4639)). Within certain embodiments, the alphavirus vector particle is from at least a portion of a Sindbis virus, Semliki Forest virus, Venezuelan equine encephalitis virus or Ross River virus.

Within other aspects, methods are provided for stimulating an immune response within a warm-blooded animal, comprising the general step of administering to a warm-blooded animal alphavirus vector particles described herein that infect dendritic cells and express an antigen or a portion thereof from a pathogenic agent. Within one

embodiment, the alphavirus vector particles are not derived entirely from Venezuelan equine encephalitis virus or Sindbis virus CMCC #4639 (deposited with the ATCC April 2, 1996: VR-2526 (however, such particles may contain a portion of VEE or CMCC #4639)). Within certain embodiments, the alphavirus vector particle is from at least a portion of a Sindbis virus, Semliki Forest virus, or Ross River virus.

Within other aspects, methods are provided for stimulating an antigen-specific T cell response, comprising the steps of infecting dendritic cells with alphavirus vector particles described herein that express an antigen from a pathogenic agent, and allowing presentation of said antigen by dendritic cells to T cells, such that said T cells are stimulated in an antigen-specific manner.

Within a further aspect of the present invention, isolated nucleic acid molecules encoding a Sindbis virus E2 glycoprotein gene are provided, wherein said nucleic acid molecule has an amino acid substitution or deletion for the wild-type glutamic acid at codon 160, and with the proviso that said glycoprotein gene is not obtained from VEE or CMCC #4639. Within one embodiment, a glycine residue is substituted at codon 160. Also provided are structural protein expression cassettes comprising such nucleic acid molecules, and alphavirus vector particles containing the above noted E2 structural protein.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth herein which describe in more detail certain procedures or compositions (*e.g.*, plasmids, etc.), and are therefore incorporated by reference in their entirety.

Brief Description Of The Drawings

Figure 1 is a schematic illustration of one representative flow chart of a human dendritic cell adaptation strategy for alphaviruses.

Figure 2A describes a general cloning strategy for the genome of a human dendritic cell adapted alphavirus (*e.g.*, Sindbis virus) variant.

Figure 2B is the nucleotide sequence of SinDCChiron virus

Figure 2C is the nucleotide sequence of SinChironLP virus

Figure 3 shows Sindbis-GFP vector infection of human dendritic cells.

Figure 4 shows increased efficiency of human DC infection by a SIN-GFP vector with SinDCChiron-derived envelope glycoproteins, as compared to glycoproteins from another DC variant of Sindbis virus.

Figure 5 shows that SIN-GFP vectors with DC adapted glycoproteins specifically target immature human dendritic cells as compared to mature DCs.

Figure 6 shows laser cytometric analysis of DC variant SIN-GFP vector infected cells trafficking to the lymph node after administration to mice

Figure 7 shows microscopy of DC variant SIN-GFP vector infected cells that have trafficked to the lymph node after administration to mice

Figure 8 is a graph showing that alphaviruses (*e.g.*, SFV or SIN-LP) are capable of infecting murine DC, but not human DC unless they are first modified or adapted for the efficient infection of such human DC (see for example SIN DC+).

Figure 9 is a graph showing alphavirus (*e.g.*, SIN) vector induced maturation and activation of human DC following transduction in vitro and murine DC following transduction in vivo.

Figure 10 shows the utility of human DC-adapted alphavirus vectors for in vitro assays that measure antigen presentation by transduced DC and stimulation of immune cells (*e.g.*, T cells).

Figure 11 is a graph comparing immune induction using recombinant alphavirus particles containing either the new SINCR replicon or a wild-type SINBV replicon.

Figure 12 is a graph that shows CTL enhancement by selected prime/boost vaccine strategies.

Figure 13 demonstrates alphavirus vector particle production from LP and DC+ derived packaging cell lines.

Figure 14 graphically depicts cellular and humoral immune responses in primates after immunization with an ELVIS vector prime and a recombinant protein boost.

Detailed Description Of The Invention

Definitions

Prior to setting forth the invention, it may be helpful to an understanding thereof to first set forth definitions of certain terms that will be used hereinafter.

An "isolated nucleic acid molecule" is a nucleic acid molecule that is not integrated in the genomic DNA of an organism, or, in the case of the virus, not contained within the genome of a wild-type virus. One example of an isolated nucleic acid molecule is a chemically-synthesized nucleic acid molecule, or, a nucleic acid molecule that is produced by recombinant (*e.g.*, PCR) techniques.

"Genomic RNA" refers to an RNA that contains all of the genetic information required to direct its own amplification or self-replication *in vivo*, within a target cell. An alphavirus-derived genomic RNA molecule should contain the following ordered elements: 5' viral or defective-interfering RNA sequence(s) required in *cis* for replication, sequences which, when expressed, code for biologically active alphavirus nonstructural proteins (*e.g.*, nsP1, nsP2, nsP3, nsP4), 3' viral sequences required in *cis* for replication, and a polyadenylate tract. The alphavirus-derived genomic RNA vector replicon also may contain a viral subgenomic "junction region" promoter and sequences which, when expressed, code for biologically active alphavirus structural proteins (*e.g.*, C, E3, E2, 6K, E1). Generally, the term genomic RNA refers to a molecule of positive polarity, or "message" sense, and the genomic RNA may be of length different from that of any known, naturally-occurring alphavirus.

"Alphavirus vector construct" refers to an assembly which is capable of directing the expression of a sequence or gene of interest. Such vector constructs are comprised of a 5' sequence which is capable of initiating transcription of an alphavirus RNA (also

referred to as 5' CSE, in background), as well as sequences which, when expressed, code for biologically active alphavirus nonstructural proteins (*e.g.*, nsP1, nsP2, nsP3, nsP4), an alphavirus RNA polymerase recognition sequence (also referred to as 3' CSE, in background), and a polyadenylate tract. In addition, the vector construct may include a viral subgenomic "junction region" promoter, sequences from one or more structural protein genes or portions thereof, extraneous nucleic acid molecule(s) which are of a size sufficient to allow production of viable virus, a 5' promoter which is capable of initiating the synthesis of viral RNA from cDNA *in vitro* or *in vivo*, a heterologous sequence to be expressed, and one or more restriction sites for insertion of heterologous sequences.

"Alphavirus RNA vector replicon", "RNA vector replicon" and "replicon" refers to an RNA molecule which is capable of directing its own amplification or self-replication *in vivo*, within a target cell. An alphavirus-derived RNA vector replicon should contain the following ordered elements: 5' viral sequences required in *cis* for replication (also referred to as 5' CSE, in background), sequences which, when expressed, code for biologically active alphavirus nonstructural proteins (*e.g.*, nsP1, nsP2, nsP3, nsP4), 3' viral sequences required in *cis* for replication (also referred to as 3' CSE, in background), and a polyadenylate tract. The alphavirus-derived RNA vector replicon also may contain a viral subgenomic "junction region" promoter, sequences from one or more structural protein genes or portions thereof, extraneous nucleic acid molecule(s) which are of a size sufficient to allow production of viable virus, as well as heterologous sequence(s) to be expressed.

"Recombinant Alphavirus Particle, Alphavirus Vector Particle" refers to a virion-like structural unit containing an alphavirus RNA vector replicon. Generally, a recombinant alphavirus particle comprises one or more alphavirus structural proteins, a lipid envelope and an RNA vector replicon. Preferably, the recombinant alphavirus particle contains a nucleocapsid structure that is contained within a host cell-derived lipid bilayer, such as a plasma membrane, in which alphaviral-encoded envelope glycoproteins are embedded. Use of the term "recombinant" when referring to alphavirus particles means that the alphavirus particles that have been generated or modified by molecular genetic manipulation, and is does not refer to wild-type

alphaviruses as found in nature. Recombinant alphavirus particles however may be derived from alphaviruses found in nature, when all or a portion of such an alphavirus is used to construct or otherwise generate a recombinant alphavirus particle.

"Alphavirus packaging cell line" refers to a cell which contains an alphavirus structural protein expression cassette and which produces recombinant alphavirus particles after introduction of an alphavirus vector construct, RNA vector replicon, eukaryotic layered vector initiation system, or recombinant alphavirus particle. The parental cell may be of mammalian or non-mammalian origin. Within preferred embodiments, the packaging cell line is stably transformed with the structural protein expression cassette.

"Eukaryotic Layered Vector Initiation System" or "ELVIS" refers to an assembly which is capable of directing the expression of a sequence(s) or gene(s) of interest. The eukaryotic layered vector initiation system should contain a 5' promoter which is capable of initiating *in vivo* (*i.e.*, within a cell) the synthesis of RNA from cDNA, and a viral vector sequence which is capable of directing its own replication in a eukaryotic cell and also expressing a heterologous sequence. In preferred embodiments, the nucleic acid vector sequence is an alphavirus-derived sequence and is comprised of a 5' sequence which is capable of initiating transcription of an alphavirus RNA (also referred to as 5' CSE, in background), as well as sequences which, when expressed, code for biologically active alphavirus nonstructural proteins (*e.g.*, nsP1, nsP2, nsP3, nsP4), and an alphavirus RNA polymerase recognition sequence (also referred to as 3' CSE, in background). In addition, the vector sequence may include a viral subgenomic "junction region" promoter, sequences from one or more structural protein genes or portions thereof, extraneous nucleic acid molecule(s) which are of a size sufficient to allow optimal amplification, a heterologous sequence to be expressed, one or more restriction sites for insertion of heterologous sequences, as well as a polyadenylation sequence. The eukaryotic layered vector initiation system may also contain splice recognition sequences, a catalytic ribozyme processing sequence, a nuclear export signal, and a transcription termination sequence.

"Antigen" refers to a molecule containing one or more epitopes (either linear, conformational or both) that will stimulate a host's immune system to make a humoral and/or cellular antigen-specific response. The term is used interchangeably with the term "immunogen." Normally, a B-cell epitope will include at least about 5 amino acids but can be as small as 3-4 amino acids. A T-cell epitope, such as a CTL epitope, will include at least about 7-9 amino acids and a helper T-cell epitope at least about 12-20 amino acids. Normally, an epitope will include between about 7 and 15 amino acids, such as, 9, 10, 12 or 15 amino acids. The term "antigen" denotes both subunit antigens, (*i.e.*, antigens which are separate and discrete from a whole organism with which the antigen is associated in nature), as well as, killed, attenuated or inactivated bacteria, viruses, fungi, parasites or other microbes. Antibodies such as anti-idiotype antibodies, or fragments thereof, and synthetic peptide mimotopes, which can mimic an antigen or antigenic determinant, are also captured under the definition of antigen as used herein. Similarly, an oligonucleotide or polynucleotide which expresses an antigen or antigenic determinant *in vivo*, such as in gene therapy and DNA immunization applications, is also included in the definition of antigen herein.

For purposes of the present invention, antigens can be derived from any of several known viruses, bacteria, parasites and fungi, as described more fully below. The term also intends any of the various tumor antigens. "Native Antigens" refers to antigens derived from natural sources, i.e. non-recombinant methods. Furthermore, for purposes of the present invention, an "antigen" refers to a protein or peptide which includes modifications, such as deletions, additions and substitutions (generally conservative in nature), to the native sequence, so long as the protein maintains the ability to elicit an immunological response, as defined herein. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts which produce the antigens.

An "immune response" to an antigen or composition is the development in a subject of a humoral and/or a cellular immune response to an antigen present in the composition of interest. For purposes of the present invention, a "humoral immune response" refers to an immune response mediated by antibody molecules, while a "cellular immune response" is one mediated by T-lymphocytes and/or other white blood cells.

One important aspect of cellular immunity involves an antigen-specific response by cytolytic T-cells ("CTL"s). CTLs have specificity for peptide antigens that are presented in association with proteins encoded by the major histocompatibility complex (MHC) and expressed on the surfaces of cells. CTLs help induce and promote the destruction of intracellular microbes, or the lysis of cells infected with such microbes. Another aspect of cellular immunity involves an antigen-specific response by helper T-cells. Helper T-cells act to help stimulate the function, and focus the activity of, nonspecific effector cells against cells displaying peptide antigens in association with MHC molecules on their surface. A "cellular immune response" also refers to the production of cytokines, chemokines and other such molecules produced by activated T-cells and/or other white blood cells, including those derived from CD4+ and CD8+ T-cells.

A composition or vaccine that elicits a cellular immune response may serve to sensitize a vertebrate subject by the presentation of antigen in association with MHC molecules at the cell surface. The cell-mediated immune response is directed at, or near, cells presenting antigen at their surface. In addition, antigen-specific T-lymphocytes can be generated to allow for the future protection of an immunized host.

The ability of a particular antigen to stimulate a cell-mediated immunological response may be determined by a number of assays, such as by lymphoproliferation (lymphocyte activation) assays, CTL cytotoxic cell assays, or by assaying for T-lymphocytes specific for the antigen in a sensitized subject. Such assays are well known in the art. See, *e.g.*, Erickson et al., *J. Immunol. 151*:4189-4199, 1993; Doe et al., *Eur. J. Immunol. 24*:2369-2376, 1994. Recent methods of measuring cell-mediated immune response include measurement of intracellular cytokines or cytokine secretion by T-cell populations, or by measurement of epitope specific T-cells (*e.g.*, by the tetramer technique)(reviewed by McMichael, A.J., and O'Callaghan, C.A., *J. Exp. Med. 187*(9):1367-1371, 1998; Mcheyzer-Williams, M.G. et al., *Immunol. Rev. 150*:5-21, 1996; Lalvani, A. et al., *J. Exp. Med. 186*:859-865, 1997).

Thus, an immunological response as used herein may be one which stimulates the production of CTLs, and/or the production or activation of helper T- cells. The antigen

of interest may also elicit an antibody-mediated immune response. Hence, an immunological response may include one or more of the following effects: the production of antibodies by B-cells; and/or the activation of suppressor T-cells and/or T-cells directed specifically to an antigen or antigens present in the composition or vaccine of interest. These responses may serve to neutralize infectivity, and/or mediate antibody-complement, or antibody dependent cell cytotoxicity (ADCC) to provide protection to an immunized host. Such responses can be determined using standard immunoassays and neutralization assays known in the art.

An "Immunogenic Composition" as used herein and as claimed refers to one or more individual components used to elicit an immune response in an animal. For example, and not intended as a limitation, the immunogenic compositions of the present invention may provide a first immunizing component selected from the group consisting of: native antigens, recombinant antigens, non-alphavirus viral vectors, nucleic acids, and alphavirus derived vector systems selected from the group consisting of alphavirus vector particles, eukaryotic layered vector initiation systems, and RNA vector replicons and a second immunizing component selected from the group consisting of: native antigens, recombinant antigens, non-alphavirus viral vectors, nucleic acids, and alphavirus derived vector systems selected from the group consisting of alphavirus vector particles, eukaryotic layered vector initiation systems, and RNA vector replicons. Providing that the first immunizing component is different from the second immunizing component and that the first immunizing component, the second immunizing component or both are alphavirus derived vector systems. Furthermore, the immunizing components that make up the immunogenic compositions of the present invention are administered to the host individually. The first immunizing component is generally administered to prime the immune system, whereas the second immunizing component is administered to boost the immune system. However, in other embodiments of the present invention the individual immunizing components may be administered simultaneously. In addition, it should be appreciated that in those instances wherein the first and second immunizing components of an immunogenic composition are both alphavirus vectors (e.g., vector particles, ELVIS, RNA replicons), there may be particular advantages to using

alphavirus vectors derived from different alphaviruses. For example, a method for inducing an immune response in an animal wherein the first immunizing component is derived from a first alphavirus and the second immunizing component is derived from a second alphavirus different from said first alphavirus is expected to minimize any deleterious effects of anti-vector immune responses that resulted from administration of the first component.

"Immature Dendritic Cells" refers to a population of cells with particular characteristics as outlined below. The criteria for identifying immature dendritic cells, both in cytokine-driven culture and from primary tissues, include their dendritic morphology, motility, phagocytic activity. Immature dendritic cells express surface CD1a, moderate expression of surface MHC and costimulatory molecules (e.g. CD40, CD80, CD86) and are negative for CD3, CD19, CD20 and CD83 (Banchereau and Steinman, *Nature 392*, 245-252, 1998). These cells have the capacity to differentiate into mature immunostimulatory antigen presenting cells, both dendritic cells and macrophages, the latter being generated via a CD14+ cell intermediate (Cella et al. *Curr Opin in Immunol. 9*:10-16, 1997). Mature dendritic cells (CD86+, MHC+ and CD83+, CD14-) and macrophages (CD14+, MHC+ c-fms+,CD83-) can present processed antigens to immune cells and are thus referred to as professional antigen-presenting cells (Hart, *Blood 90*: 3245-3287, 1997).

"Infects human dendritic cells" refers to recombinant alphavirus particles or alphaviruses that efficiently infect or transduce human dendritic cells. In this context, transduction or infection efficiency refers to the ability of a recombinant alphavirus particle or alphavirus to bind to, penetrate and deliver an RNA vector replicon or alphavirus genome to the cytoplasm of a host cell, thereby allowing replicon mediated expression of a heterologous sequence (*e.g.*, a reporter or antigen), or genome-mediated expression of structural proteins in a host cell. Generally, recombinant alphavirus particles or alphaviruses will efficiently transduce or infect host cells in vitro at 10, 20, 25, or 50 fold over a wild-type alphavirus of the same species at an MOI of 100 or less. Alternatively, transduction or infection efficiency is at least 20, 30, 40, or 50% of cells when performed in vitro at an MOI of 100 or less. Wild-type alphaviruses for comparison, as well as cDNA clones for use in the construction of corresponding

replicons and structural protein expression cassettes, can be obtained by using full-length virus clones referenced in the following publications: Sindbis virus - Rice et al., 1987, J. Virol. 61:3809-3819; Semliki Forest virus - Liljestrom et al., 1991, J. Virol. 65:4107-4113; Ross River virus - Kuhn et al., 1991, Virology 182:430-441; Venezuelan equine encephalitis virus - Davis et al., 1989, Virology 171:189-204.

"Isolated alphaviruses" refers to alphaviruses that are separated from the cells in which they are propagated. Alphaviruses may be further purified by a number of techniques, including for example, plaque purification.

As noted above, the present invention provides compositions and methods suitable for generating an immune response in a warm-blooded animal (*e.g.*, a human, horse, cow, sheep, pig, dog, cat, rat or mouse), comprising the general step of administering to an animal a gene delivery vector (*e.g.*, alphavirus vector particles, ELVIS, DNA), or, protein followed by the step of administering to the same animal the same or similar antigen by a second gene delivery vector (*e.g.* alphavirus vector particles, ELVIS, or, DNA), or, protein, wherein the first and second methods are different. By administering two separate gene delivery vectors (or a vector and protein), one can boost the immune response of the warm-blooded animal to the desired antigen.

In order to further an understanding of the present invention, described in more detail below are (A) methods for generating alphavirus-based gene delivery vectors and DNA-based gene delivery vectors; and (B) methods of utilizing one or both in a variety of methods.

A. Alphavirus-based gene delivery vectors, and DNA-based gene delivery vectors.

A wide variety of alphavirus-based gene delivery vectors may be readily generated using the disclosure provided herein. Representative examples of such vectors include RNA vector replicons, alphavirus vector constructs, and recombinant alphavirus particles. Briefly, sequences encoding wild-type alphaviruses suitable for use in preparing the above-described vectors can be readily obtained from naturally-occurring sources, or from depositories (*e.g.*, the American Type Culture Collection, Rockville, Maryland). In addition, wild-type alphaviruses may be utilized for comparing

their ability to infect dendritic cells, macrophages or antigen presenting cells with the alphaviruses and derived vectors of the present invention.

Representative examples of suitable alphaviruses include Aura virus (ATCC VR-368), Bebaru virus (ATCC VR-600, ATCC VR-1240), Cabassou virus (ATCC VR-922), Chikungunya virus (ATCC VR-64, ATCC VR-1241), Eastern equine encephalomyelitis virus (ATCC VR-65, ATCC VR-1242), Fort Morgan virus (ATCC VR-924), Getah virus (ATCC VR-369, ATCC VR-1243), Kyzylagach virus (ATCC VR-927), Mayaro virus (ATCC VR-66, ATCC VR-1277), Middleburg virus (ATCC VR-370), Mucambo virus (ATCC VR-580, ATCC VR-1244), Ndumu virus (ATCC VR-371), Pixuna virus (ATCC VR-372, ATCC VR-1245), Ross River virus (ATCC VR-373, ATCC VR-1246), Semliki Forest virus (ATCC VR-67, ATCC VR-1247), Sindbis virus (ATCC VR-68, ATCC VR-1248; *see also* CMCC #4640, described below), Tonate virus (ATCC VR-925), Triniti virus (ATCC VR-469), Una virus (ATCC VR-374), Venezuelan equine encephalomyelitis virus (ATCC VR-69, ATCC VR-923, ATCC VR-1250 ATCC VR-1249, ATCC VR-532), Western equine encephalomyelitis virus (ATCC VR-70, ATCC VR-1251, ATCC VR-622, ATCC VR-1252), Whataroa virus (ATCC VR-926), and Y-62-33 virus (ATCC VR-375).

Representative examples of suitable methods for constructing alphavirus-based vector systems are described in more detail in PCT Publication No. WO 97/38087 entitled "Recombinant Alphavirus-Based Vectors With Reduced Inhibition Of Cellular Macromolecular Synthesis" and U.S. Patent Nos. 5,091,309 and 5,217,879, 5,843,723, and 5,789,245.

Within one aspect of the invention, a variety of expression cassettes are provided, which contain the sequences coding for and operably express one or more alphavirus structural polypeptides provided herein. Generally, the expression cassettes fall within one of three categories: 1) a DNA promoter of RNA transcription (*e.g.*, RNA polymerase II promoter) directly and operably linked to the structural protein open reading frame (ORF), and a transcription termination/polyadenylation sequence; 2) an alphavirus defective helper RNA transcribed *in vitro* or *in vivo*, comprising the ordered elements 5' viral or defective-interfering RNA sequence required in *cis* for alphaviral

replication (also referred to as 5' CSE, in background), viral subgenomic junction region promoter, alphavirus structural protein sequence of the present invention, 3' alphaviral sequence required in *cis* for replication (also referred to as 3' CSE, in background), and polyadenylate tract; and 3) DNA cassettes comprising the ordered elements of a DNA promoter of RNA transcription that functions within a eukaryotic cell (*e.g.*, RNA polymerase II promoter) and is operably linked to a 5' viral or defective-interfering RNA sequence required in *cis* for alphaviral replication, viral subgenomic junction region promoter, alphavirus structural protein sequence of the present invention, 3' alphaviral sequence required in *cis* for replication, polyadenylate tract, and transcription termination/polyadenylation sequence. In preferred embodiments, the structural proteins of the present invention are synthesized at high levels by the cassettes only after induction by the RNA vector replicon itself or some other provided stimulus. In further embodiments, the structural protein expression cassettes do not express all alphavirus nonstructural proteins.

Within further aspects of the invention, alphavirus packaging cell lines are provided. In particular, within one aspect of the present invention, alphavirus packaging cell lines are provided wherein the viral structural proteins are supplied in *trans* from one or more stably transformed expression cassettes, and are able to encapsidate transfected, transduced, or intracellularly produced alphavirus vector RNA transcripts in the cytoplasm and release functional packaged vector particles through the plasma membrane. In preferred embodiments, the structural proteins necessary for packaging are synthesized at high levels only after induction by the RNA vector replicon itself or some other provided stimulus, and the transcripts encoding these structural proteins are capable of cytoplasmic amplification in a manner that will allow expression levels sufficient to mimic that of a natural viral infection (WO 97/38087 and U.S. Patent 5,789,245). In other embodiments, the structural proteins may be specifically modified prior to use in alphavirus packaging cell lines (*e.g.*, sequence coding for the nuclear localization signal of VEE capsid protein may be altered by site-directed mutagenesis to prevent this function). Furthermore, in other embodiments, expression of a selectable marker is operably linked to the structural protein

expression cassette. Such a linked selectable marker allows efficient generation of stable packaging cell lines.

As provided by the invention, methods for generation (packaging) of recombinant alphavirus vector particles are provided and may be readily accomplished for example, by co-transfection of complementing vector and defective helper (DH) molecules as *in vitro* transcribed RNA or plasmid DNA, or by co-infection with virus (see Xiong et al., *Science 243*:1188-1191, 1989, Bredenbeek et al., *J. Virol. 67*:6439-6446, 1993, Dubensky et al., *J. Virol 70*:508-519, 1996 and U.S. Patents 5,814,482, 5,739,026, 5,766,602, 5,789,245 and 5,792,462. Alternatively, vector particles may be generated by introduction of vector RNA into stable alphavirus packaging cell lines or "PCL" (Polo et al., PNAS 96:4598-4603; U.S. Patent 5,789,245). Briefly, such PCL and their stably transformed structural protein expression cassettes can be derived using methods described within U.S. Patent 5,789,245, or using novel methods described within this invention. For example, the production of recombinant alphavirus vector particles by PCL may be accomplished following introduction of alphavirus-based vector molecules into the PCL, the vectors being derived from *in vitro* transcribed RNA, plasmid DNA, or previously obtained recombinant alphavirus particles, incubating the PCL for a under conditions and for a time necessary for vector particle packaging, and harvesting of the packaged vector particles. Packaging cell lines may be used for the serial propagation of alphavirus vector particles following high or low multiplicity of infection.

In addition to the above viral-based vectors, numerous non-viral gene delivery vehicles may likewise be utilized within the context of the present invention. Representative examples of such gene delivery vehicles include direct delivery of nucleic acid expression vectors or naked DNA alone (see *e.g.*, U.S. Patent Nos. 5,814,482 and 5,580,859),

B. Use of gene therapy vectors

As noted above, within one aspect of the present invention methods are provided for generating or enhancing an immune response utilizing one or more gene delivery vectors (*e.g.*, alphavirus or non-alphavirus viral vectors) and/or recombinant protein in

order to prime and boost an immune response. Representative examples of such disease targets include viral infections such as HIV, HBV, HCV, HTLV I, HTLV II, CMV, EBV, HSV, respiratory syncytial virus, HPV, as well as cancer antigens (*e.g.*, melanoma). More specifically, within one aspect of the present invention, compositions and methods are provided for stimulating an immune response (either humoral or cell-mediated) to a pathogenic agent, such that the pathogenic agent is either killed or inhibited. Representative examples of pathogenic agents include bacteria, fungi, parasites, viruses and cancer cells.

This approach presents a distinct advantage over others since the antigenic epitopes expressed can be altered by selective cloning of sub-fragments of the gene for the antigen into the alphavirus vector, leading to responses against immunogenic epitopes which may otherwise be overshadowed by immunodominant epitopes. Such an approach may be extended to the expression of a peptide having multiple epitopes, one or more of the epitopes being derived from different proteins. Further, this aspect of the invention allows efficient stimulation of cytotoxic T lymphocytes (CTL) directed against antigenic epitopes, and peptide fragments of antigens encoded by sub-fragments of genes, through intracellular synthesis and association of these peptide fragments with MHC Class I molecules. This approach may be utilized to map major immunodominant epitopes for CTL induction.

An immune response may also be achieved by transferring to an appropriate immune cell (such as a T lymphocyte) the gene for the specific T cell receptor which recognizes the antigen of interest (in the context of an appropriate MHC molecule if necessary), for an immunoglobulin which recognizes the antigen of interest, or for a hybrid of the two which provides a CTL response in the absence of the MHC context. Thus, the gene delivery vehicle cells may be used as an immunostimulant, immunomodulator, or vaccine.

One particularly appealing use for alphavirus vectors is human vaccine and therapeutic application. As described in the detailed description and examples below, the present invention provides compositions and methods to increase the potency of such alphavirus-based systems, particularly in the area of vaccines. One approach

exemplified by the present invention is the targeting of alphavirus vectors directly to antigen presenting cells (*e.g.*, dendritic cells, macrophages). It is known that among the many members of the alphavirus genus, Venezuelan equine encephalitis (VEE) virus is naturally lymphotropic, while the other members are not (Caley et al., *J. Virol.* *71*:3031-3038, 1997). Yet it has also been observed that one member of the alphavirus group, VEE (MacDonald et al., J. Virol. 74:914-922), and now SFV and SIN (Figure 8) are capable of infecting murine dendritic cells at relatively high efficiency. Unfortunately, the ability of an alphavirus to efficiently infect murine dendritic cells is not predictive of its ability to efficiently infect human dendritic cells (Figure 8), which would be a goal of vaccine optimization for human use with these vectors. It should also be noted that VEE virus is a significant human pathogen responsible for sporadic epidemic outbreaks of encephalitis, resulting in multiple deaths or permanent central nervous system sequelae in virus infected individuals. Thus, VEE is classified for use under BL-3 level containment, suggesting that its development as a vaccine vector is somewhat dubious.

As illustrated below, alphaviruses, including those considered to be non-lymphotropic alphaviruses (*see* Caley et al., *ibid*) may be modified or adapted for efficient infection of human dendritic cells, their genomes then cloned, sequenced, and genetic modifications responsible then used in the construction of vectors and packaging cassettes useful for gene delivery to human dendritic cells. It should also be noted that this method of modification or adaptation to specifically target desired human cell types or populations is not limited to dendritic cells. Rather, the method can be extended to other relevant target cell types (*e.g.*, cancer cells) by using the same process of cycling virus or vector through primary cultured cells, followed by cloning and construction of vectors and packaging cassettes. The invention also provides additional strategies to enhance the vaccine efficacy of alphavirus-based vectors, by utilizing prime-boost regimes that comprise administering or expressing a desired antigen using two separate and different methods, selected for example from alphavirus vector particles, nucleic acid (*e.g.*, DNA or ELVIS vectors), recombinant protein, non-alphavirus viral vectors (e.g., poxvirus) and combined in any variety of

order, provided that either the prime or the boost or both is an alphavirus derived vector.

Administration of the vectors and/or recombinant proteins of the present invention to a vaccinee, for example in the prime/boost strategy, may be accomplished by a variety of routes. For example, within one embodiment a warm-blooded animal may be primed by a first method (*e.g.*, alphavirus vector particles, ELVIS, DNA, non-alphavirus viral vector, or recombinant protein) that stimulates an immune response against the desired antigen(s). A wide variety of inoculation routes may be utilized in this regard, including for example, intraocularly, intranasally, sublingually, orally, topically, intravesically, intrathecally, intravaginally, intrarectally topically, intravenously, intraperitoneally, intracranially, intramuscularly, intradermally, or, subcutaneously. Priming by the first method may include multiple administrations of the selected method. Following priming, the same or substantially the same antigen or a modified form thereof is administered to the same animal via a second method (*e.g.*, alphavirus vector particles, ELVIS, DNA, non-alphavirus viral vector, or recombinant protein) that stimulates an immune response against the desired antigen(s). Similar to the priming step, a wide variety of inoculation routes may be utilized in this regard (see above list). Within certain embodiments, the boosting step may be performed several times, as is best suited to generate a protective or therapeutic immune response.

Within yet other aspects of the present invention, the above-described alphavirus-based vector system (*e.g.*, alphavirus vector particles, RNA vector replicons, eukaryotic layered vector initiation systems and the like) may be used for non-vaccine therapeutics, such as the treatment of coronary artery disease (CAD) and/or peripheral vascular disease (PVD). For example, within one embodiment an alphavirus-based vector such as ELVIS or recombinant alphavirus particle can be administered to a patient to treat or prevent CAD or PVD. In cardiovascular indications, including CAD and PVD, alphavirus vectors, both DNA- and particle-based can be used to deliver particular palliatives which stimulate angiogenesis and/or neovascularization. Included among these palliatives are, for example, fibroblast growth factor (*e.g.*, FGF 1, 2, 4, 5, 18), vascular endothelial growth factor (*e.g.*, VEGF-

2, D), endothelial nitric oxide synthetase (*e.g.*, NOS). Delivery of these vectors for therapeutic effect can be accomplished by several alternative routes, including for example intramuscular for PVD, and intracoronary, intramyocardial, or perivascular, for CAD.

Adjuvants may also be used to enhance the effectiveness of the vaccinating compositions used for priming or boosting. Such adjuvants include, but are not limited to: (1) aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, aluminum sulfate, etc.; (2) oil-in-water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides (see below) or bacterial cell wall components), such as for example (a) MF59 (International Publication No. WO 90/14837), containing 5% Squalene, 0.5% Tween 80, and 0.5% Span 85 (optionally containing various amounts of MTP-PE (see below), although not required) formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer (Microfluidics, Newton, MA), (b) SAF, containing 10% Squalane, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP (see below) either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (c) Ribi™ adjuvant system (RAS), (Ribi Immunochem, Hamilton, MT) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox™); (3) saponin adjuvants, such as Stimulon™ (Cambridge Bioscience, Worcester, MA) may be used or particle generated therefrom such as ISCOMs (immunostimulating complexes); (4) Complete Freunds Adjuvant (CFA) and Incomplete Freunds Adjuvant (IFA); (5) cytokines, such as interleukins (IL-1, IL-2, etc.), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), etc.; (6) oligonucleotides or polymeric molecules encoding immunostimulatory CpG motifs (Davis, H.L., et al., *J. Immunology 160*:870-876, 1998; Sato, Y. et al., *Science 273*:352-354, 1996) or complexes of antigens/oligonucleotides. Polymeric molecules include double and single stranded RNA and DNA, and backbone modifications thereof, for example, methylphosphonate linkages. Further, such polymeric molecules include alternative polymer backbone structures such as, but not limited to, polyvinyl backbones (Pitha,

Biochem Biophys Acta 204:39, 1970a; Pitha, *Biopolymers* 9:965, 1970b), and morpholino backbones (Summerton, J., et al., U.S. Patent No. 5,142,047, issued 08/25/92; Summerton, J., *et al.*, U.S. Patent No. 5,185,444 issued 02/09/93). A variety of other charged and uncharged polynucleotide analogs have been reported. Numerous backbone modifications are known in the art, including, but not limited to, uncharged linkages (*e.g.*, methyl phosphonates, phosphotriesters, phosphoamidates, and carbamates) and charged linkages (*e.g.*, phosphorothioates and phosphorodithioates).}; and (7) other substances that act as immunostimulating agents to enhance the effectiveness of the VLP immune-stimulating (or vaccine) composition. Alum, CpG oligonucleotides, and MF59 are preferred.

A variety of genes encoding polypeptide antigens can be used in the practice of the present invention. Antigens can be derived from a wide variety of viruses, bacteria, fungi, protozoans and other parasites. For example, the present invention will find use for stimulating an immune response against a wide variety of proteins or peptides from the herpes virus family, including proteins derived from herpes simplex virus (HSV) types 1 and 2, such as HSV-1 and HSV-2 gB, gD, gH, VP16 and VP22; antigens derived from varicella zoster virus (VZV), Epstein-Barr virus (EBV) and cytomegalovirus (CMV) including CMV gB and gH; and antigens derived from other human herpesviruses such as HHV6 and HHV7. (See, *e.g.* Chee et al., *Cytomegaloviruses* (J.K. McDougall, ed., Springer-Verlag 1990) pp. 125-169, for a review of the protein coding content of cytomegalovirus; McGeoch et al., *J. Gen. Virol.* 69:1531-1574, 1988, for a discussion of the various HSV-1 encoded proteins; U.S. Patent No. 5,171,568 for a discussion of HSV-1 and HSV-2 gB and gD proteins and the genes encoding therefor; Baer et al., *Nature* 310:207-211, 1984, for the identification of protein coding sequences in an EBV genome; and Davison and Scott, *J. Gen. Virol.* 67:1759-1816, 1986, for a review of VZV.)

Additionally, immune responses to antigens from viruses associated with hepatitis, including hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), the delta hepatitis virus (HDV), hepatitis E virus (HEV), and hepatitis G virus, can also be stimulated using the constructs of the present invention. By way of example, the HCV genome encodes several viral proteins, including the structural core, E1 (also known

as E) and E2 (also known as E2/NSI) proteins, as well as nonstructural proteins (*e.g.*, NS3, NS4, NS5), which will find use with the present invention (see, Houghton et al. *Hepatology 14*:381-388, 1991, for a discussion of HCV proteins, including E1 and E2). The delta-antigen from HDV can also be used (see, *e.g.*, U.S. Patent No. 5,389,528, for a description of the delta-antigen).

Similarly, influenza virus is another example of a virus for which the present invention will be particularly useful. Specifically, the envelope glycoproteins HA and NA of influenza A are of particular interest for generating an immune response. Numerous HA subtypes of influenza A have been identified (Kawaoka et al., *Virology 179*:759-767, 1990; Webster et al. "Antigenic variation among type A influenza viruses," p. 127-168. In: P. Palese and D.W. Kingsbury (ed.), *Genetics of influenza viruses*. Springer-Verlag, New York).

Other antigens of particular interest to be used in the practice of the present invention include antigens and polypeptides derived therefrom from human papillomavirus (HPV), such as one or more of the various early proteins including E6 and E7; tick-borne encephalitis viruses; and HIV-1 (also known as HTLV-III, LAV, ARV, etc.), including, but not limited to, antigens such as gp120, gp41, gp160, Gag and pol from a variety of isolates including, but not limited to, HIV_{IIIb}, HIV_{SF2}, $HIV\text{-}1_{SF162}$, $HIV\text{-}1_{SF170}$, HIV_{LAV}, HIV_{LAI}, HIV_{MN}, $HIV\text{-}1_{CM235}$,, $HIV\text{-}1_{US4}$, other HIV-1 strains from diverse subtypes (*e.g.*, subtypes, A through G, and O), HIV-2 strains and diverse subtypes (*e.g.*, $HIV\text{-}2_{UC1}$ and $HIV\text{-}2_{UC2}$). See, *e.g.*, Myers, et al., Los Alamos Database, Los Alamos National Laboratory, Los Alamos, New Mexico; Myers, et al., *Human Retroviruses and Aids, 1990*, Los Alamos, New Mexico: Los Alamos National Laboratory.

Proteins derived from other viruses will also find use in the methods described herein, such as for example, proteins from members of the families Picornaviridae (*e.g.*, polioviruses, etc.); Caliciviridae; Togaviridae (*e.g.*, rubella virus, dengue virus, etc.); Flaviviridae; Coronaviridae; Reoviridae; Birnaviridae; Rhabdoviridae (*e.g.*, rabies virus, etc.); Filoviridae; Paramyxoviridae (*e.g.*, mumps virus, measles virus, respiratory syncytial virus, etc.); Orthomyxoviridae (*e.g.*, influenza virus types A, B and C, etc.);

Bunyaviridae; Arenaviridae; Retroviradae, *e.g.*, HTLV-I; HTLV-II; HIV-1; HIV-2; simian immunodeficiency virus (SIV) among others. See, *e.g.*, Virology, 3rd Edition (W.K. Joklik ed. 1988); *Fundamental Virology*, 2nd Edition (B.N. Fields and D.M. Knipe, eds. 1991; *Virology*, 3rd Edition (Fields, BN, DM Knipe, PM Howley, Editors, 1996, Lippincott-Raven, Philadelphia, PA) for a description of these and other viruses.

Particularly preferred bacterial antigens are derived from organisms that cause diphtheria, tetanus, pertussis, meningitis, and other pathogenic states, including, without limitation, antigens derived from *Corynebacterium diphtheriae*, *Clostridium tetani*, *Bordetella pertusis*, *Neisseria meningitidis*, including serotypes *Meningococcus* A, B, C, Y and WI35 (MenA, B, C, Y and WI35), *Haemophilus influenza* type B (Hib), and *Helicobacter pylori*. Examples of parasitic antigens include those derived from organisms causing malaria and Lyme disease.

Furthermore, the methods described herein provide means for treating a variety of malignant cancers. For example, the system of the present invention can be used to enhance both humoral and cell-mediated immune responses to particular proteins specific to a cancer in question, such as an activated oncogene, a fetal antigen, or an activation marker. Tumor antigens include for example any of the various MAGEs (melanoma associated antigen E), including MAGE 1, 2, 3, 4, etc. (Boon, T. *Scientific American* (March 1993):82-89); any of the various tyrosinases; MART 1 (melanoma antigen recognized by T cells), gp100, TRP-2, mutant ras; mutant p53; p97 melanoma antigen; CEA (carcinoembryonic antigen), among others.

C. Deposit Information

The following materials have been deposited with the American Type Culture Collection:

Deposit	Designation	Deposit Date	Accession No.
Wild type Sindbis virus	CMCC #4639	April 2, 1996	VR-2526

SinDCchiron virus	NA	April 13, 1999	VR-2643

The above materials were deposited by Chiron Corporation with the American Type Culture Collection (ATCC), under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for purposes of Patent Procedure. The accession number is available from the ATCC at telephone number (301) 881-2600.

These deposits are provided as convenience to those of skill in the art, and are not an admission that a deposit is required under 35 U.S.C. § 112. The nucleic acid sequence of these deposits, as well as the amino acid sequence of the polypeptides encoded thereby, are incorporated herein by reference and should be referred to in the event of an error in the sequence described therein. A license may be required to make, use, or sell the deposited materials, and no such license is granted hereby.

The following examples are included to more fully illustrate the present invention. Additionally, these examples provide preferred embodiments of the invention and are not meant to limit the scope thereof. Standard methods for many of the procedures described in the following examples, or suitable alternative procedures, are provided in widely reorganized manuals of molecular biology, such as, for example, "Molecular Cloning," Second Edition (Sambrook et al., Cold Spring Harbor Laboratory Press, 1987) and "Current Protocols in Molecular Biology" (Ausubel et al., eds. Greene Associates/Wiley Interscience, NY, 1990), as well as in U.S. Patents 6,015,686, 5,814,482, 6,015694, 5,842,723, and 5,789,245, and as such, each of these is referenced in its entirety.

EXAMPLES

Example 1

Selection and Cloning of Alphavirus Variants That Infect Primary Human Dendritic Cells

In order to demonstrate that viruses of the Alphavirus genus, including those previously characterized as non-lymphotropic or previously shown to infect murine

dendritic cells, could be modified or adapted to efficiently infect and propagate in human dendritic cells, Sindbis virus was chosen as a representative example. Other similar alphaviruses, such as Semliki Forest virus, Venezuelan equine encephalitis virus and Ross River virus, also may be readily substituted by one of skill in the art, using the disclosure provided herein. Using a naturally occurring heterogeneous virus sample that was propagated only a very limited number of cycles in common laboratory cell lines, adaptation was performed as outlined in Figure 1. Briefly, the virus was passaged 4 times in primary human dendritic cells obtained from different donors, with intermediate plaque purification in 293 and BHK-21 cells.

The primary human dendritic cells used for virus passage were derived from peripheral blood monocytes as previously described (Bender et al., *J Immunol. Meth.* *196*:121, 1996). The buffy coat population of cells was obtained from healthy donors at the Blood Center of the Pacific (San Francisco, CA) or Stanford Medical School Blood Center (Palo Alto, CA). $CD14^+$ monocytes were isolated by negative depletion using Monocyte Isolation Kits and mini-MACS columns (Miltenyi Biotec GmbH), according to the manufacturer's instructions. Dendritic cells were generated from the CD14+ cells by culturing at $0.6x10^6$ per ml in RPMI 1640 medium, supplemented with 10% FCS, 2 mM glutamine, penicillin/streptomycin, 1,000 U/ml rhGM-CSF (Peprotech), and 1,000 U/ml rhIL-4 (Peprotech). Culture medium containing cytokines was replenished every two days. Monocyte-conditioned medium (MCM) was prepared as previously described (Bender et al., *ibid*) and was added to immature DC cultures at 30% (vol/vol) to induce maturation, either 5 or 6 days following culture initiation, for 3 additional days. Expression of cell surface markers upon MCM-treatment was analyzed by flow cytometry (see Gardner et al., J. Virol., 74:11849, 2000).

Following dendritic cell adaptation, a panel of plaque-purified clonal virus variants was able to grow efficiently in primary human dendritic cells, producing virus titers of greater than 10^8 PFU/ml, or 1000 infectious virus particles/cell. Each clonal variant formed small plaques on BHK-21 and Vero cells, as compared to the parental virus and other common laboratory Sindbis virus strains. One of the DC-selected plaque-purified viruses was chosen for cDNA cloning, and an agarose plug from the last

plaque purification step was incubated in 1 ml of media (MEM with 10% FBS) for 4 hours at 4°C. A 100 ul aliquot of virus eluate then was used to infect 10^6 BHK-21 cells. After development of CPE the media (5 ml) was harvested. This virus stock produced homogeneous small plaques on both BHK-21 and Vero cells, and the titer was $2x10^8$ PFU/ml. Virus from this seed stock, designated SinDCchiron, was deposited with American Type Culture Collection according to the requirements of the Budapest Treaty.

In addition, a small number of spontaneous large plaque variants were observed sporadically in BHK-21 and Vero cells. The plaque size of these variants was significantly larger than the parental virus and other common laboratory strains of Sindbis virus. Similar to other alphaviruses, this large plaque virus variant was inefficient at infecting primary human dendritic cells and produced very low titers of progeny virus (<10^5 PFU/ml). One of the large plaque variants was chosen for cDNA cloning, and an agarose plug from the last plaque purification step was incubated in 1 ml of media (MEM with 10% FBS) for 4 hours at 4°C. A 100 ul aliquot of virus eluate then was used to infect 10^6 BHK-21 cells. After development of CPE the media (5 ml) was harvested. This virus stock produced homogeneous large plaques on both BHK-21 and Vero cells. Virus from this seed stock was designated SinChironLP.

Approximately 10^7 BHK-21 cells were infected with either the dendritic cell adapted virus seed stock or the large plaque virus seed stock at a MOI of 1 PFU/cell. At 24 hours post-infection, after development of CPE, total RNA was isolated from the cells using the TRIzol Reagent (GibcoBRL) according to the manufacturer's instructions. After purification, viral RNA was dissolved in nuclease-free water, aliquoted, and stored at –80°C for later use in cDNA cloning (Figure 2A).

Synthesis of cDNA was accomplished by PCR amplification, using the primer sets shown below (Sindbis nucleotide numbering indicated for each primer):

1	CCAC*AAGCTT*GATCTAATGTACCAGCCTGATGC	11472-11450
1.1	CCAC*GAATTC*AGCCAGATGAGTGAGGC	10364-10381
2	CCAC*AAGCTT*CAATTCGACGTACGCCTCAC	10394-10375

2.1	CCAC*GAATTC*ATATGGGGAAATCATGAGCC	9614-9634
3	CCAC*AAGCTT*CATAGACCCTCACTGGCTC	9648-9630
3.1	CCAC*GAATTC*AAGATTAGCACCTCAGGACC	8878-8899
4	CCAC*AAGCTT*CTACACGGTCCTGAGGTGC	8908-8887
4.1	CCAC*GAATTC*GTCCGATCATGGATAACTCC	8294-8315
5	CCAC*AAGCTT*GCGCCACCGAGGAC	8347-8334
5.1	CCAC*GAATTC*ACTGCCATGTGGAGGCC	7797-7814
6	CCAC*CTCGAG*TTTACCCAACTTAAACAGCC	7368-7348
6.1	CCAC*GAGCTC*GCGACATTCAATGTCGAATGC	6426-6446
7	CCAC*CTCGAG*GAACTCCTCCCAATACTCGTC	6488-6468
7.1	CCAC*GAGCTC*GACCTTGGAGCGCAATGTCC	5843-5862
8	CCAC*CTCGAG*TTTCGACGTGTCGAGCACC	5900-5882
8.1	CCAC*GAGCTC*GACCATGGAAGCAATCCGC	4814-4832
9	CCAC*CTCGAG*ACGACGGGTTATGGTCGAC	4864-4845
9.1	CCAC*GAGCTC*CACGGAGACAGGCACCGC	4246-4264
10	CCAC*CTCGAG*GATCACTTTCTTTCCTAGGCAC	4299-4277
10.1	CCAC*GAGCTC*GAACTCTCCCGTAGATTTCC	3407-3427
11	CCAC*CTCGAG*ATCAAGTTGTGTGCCCTTCC	3464-3445
11.1	CCAC*GAGCTC*CCAGGGGATATCATCCTGAC	2742-2761
12	CCAC*CTCGAG*GCTGTCATTACTTCATGTCCG	2825-2804

12.1 CCAC*GAGCTC*GAACCGCAAACTATACCACATTGC 1976-1999

13 CCAC*CTCGAG*CTTGTACTGCTCCTCTTCTG 2042-2023

13.1 CCAC*GAGCTC*GGAGAACGGGTATCGTTCC 1029-1047

14 CCAC*CTCGAG*CCGGGATGTACGTGCAC 1069-1052

14.1 CCAC*GAGCTC*ATTGACGGCGTAGTACACAC 1-20

Primer pairs 1-5 were used for cloning of the virus structural genes, while pairs 6-14 were for the virus nonstructural genes. Oligonucleotides in pairs 1-5 contained additional sequences representing restriction enzyme sites for *EcoR*I and *Hind*III, which are not present in subgenomic RNA of Sindbis virus. Oligonucleotides 6-14 contained sites for *Sac*I and *Xho*I, which are not present in the whole genome of previously sequenced strains of Sindbis virus (these sites are underlined).

Each reverse transcription (RT) reaction was performed in a 50 ul volume using the SuperscriptII enzyme (GibcoBRL), according to the manufacturer's instructions. Reaction mixtures contained the amount of RNA equivalent to 10^6 cells and 50 pmoles of each primer shown below.

Mixture1: primers1, 3 and 5

Mixture2: primers 2 and 4

Mixture3: primers 6, 9 and 12

Mixture4: primers 8, 11 and 14

RT reactions were frozen and then used directly for PCR amplification. PCR reactions were performed using Vent DNA polymerase (NEB) as recommended by the manufacturer. Each 50 ul PCR reaction contained 3 ul of RT mixtures described above and 50 pmoles of primers. A total of 14 reactions were performed (Table 1).

Table 1

N of fragment	Primers	N of RT reaction.	Length of the fragment (bp.)
1	1 and 1.1	1	1128
2	2 and 2.1	2	800
3	3 and 3.1	1	789
4	4 and 4.1	2	644
5	5 and 5.1	1	670
6	6 and 6.1	3	962
7	7 and 7.1	3	666
8	8 and 8.1	4	1107
9	9 and 9.1	3	638
10	10 and 10.1	3	912
11	11 and 11.1	4	743
12	12 and 12.1	3	870
13	13 and 13.1	3	1034
14	14 and 14.1	4	1088

PCR reactions for fragments 1-5 were performed using the following conditions: 12 cycles of 95°C for 30 seconds, 56°C for 30 seconds and 74°C for 90 seconds. For fragments 6-14, the number of cycles was changed from 12 to 15. A small aliquot of each reaction mixture was analyzed by agarose gel electrophoresis to confirm the presence of the fragments of the expected size. The rest was extracted with phenol-chloroform and DNA fragments were precipitated using ethanol.

For cloning, fragments 1-5 were digested with *Hind*III and *Eco*RI, and then ligated with plasmid pRS2 (pUC19 with additional restriction sites in polylinker) treated with the same enzymes. Fragments 6-14 were digested with *Sac*I and *Xho*I and ligated with the same pRS2 plasmid treated with *Sac*I and *Xho*I. All recombinant plasmids were transformed into the *E. coli* XL-1 Blue strain (Stratagene, La Jolla, CA).

In addition, cDNA clones representing the subgenomic promoter region and 3'-end nontranslated regions also were generated using the following primer pairs:

YSIN1F

5'-GATTCGGTTACTTCCACAGC

YSIN1R

5'-ACTGACGGCTGTGGTCAGTT

YSIN2F

5'-GATGTACTTCCGAGGAACTG

YSIN2R

5'-CCACAAGCTTGAAATGTTAAAAACAAAATTTTGT

Three positive colonies for each transformation were grown in 40 ml of 2xYT media supplemented with ampicillin (200 µg/ml), plasmids were purified using a QIAGEN kit according to the manufacturer's instructions, and the insertions were sequenced. By comparison of sequences from three independent clones, the genome sequence of each virus was determined (Figures 2B and 2C) and compared to published sequences for other Sindbis virus strains. Correct cDNA fragments for each virus (based on consensus of the three independent clones) were designated p1-p14 correspondingly.

Sequence data demonstrated that the SinDCChiron (also known as DC+) and SinChironLP (also known as LP) strains differed at only a single amino acid residue, throughout their entire genomes. This determinant for efficient infection of human

dendritic cells, and in particular immature human DC, was located at E2 glycoprotein residue 160, with the strains containing the amino acids Gly or Glu, respectively. Therefore, substitution of Gly for Glu at E2 160 alone is responsible for conferring the human DC-adapted phenotype. Additional amino acid substitutions, deletions or insertions at, or in close proximity to, this site can be readily generated by standard site-directed mutagenesis protocols, and when inserted into a full-length cDNA clone described below, also may produce the same human DC adapted growth characteristics.

Example 2

Construction of a full-length cDNA clone, vectors and packaging cassettes from Human dendritic cell adapted alphaviruses

The construction of a full-length cDNA clone, replicon vectors, structural protein expression (packaging) cassettes and stable packaging cell lines derived from any human dendritic cell tropic alphavirus (*e.g.*, SinDCchiron virus) may be readily accomplished by one of skill in the art using the teachings provided below, as well as those previous teachings provided by U.S. Patents 5,814,482, 5,789,245, 5,843,723, 6,015,686, and 6,015,694, and published PCT WO 97/38087. For example, construction of a vector replicon from clones of the human DC tropic SinDCchiron virus described above was accomplished using clones p1-p14 (Example 1) as follows. An *Apa*I-*Msc*I fragment, containing the promoter for SP6 RNA polymerase and start of the Sindbis virus genomic RNA, was ligated with the *Msc*I-*Xho*I fragment of cloned fragment 14 in *Apa*I-*Xho*I digested plasmid pRS2. The resulting plasmid was named p15. Next, the *Sac*I-*Eco*RI fragment of p8, the *Eco*RI-*Nsi*I fragment of p7 and the *Nsi*I-*Xho*I fragment of p6 were ligated into *Sac*I-*Xho*I digested pRS2. The resulting plasmid was named p16. Next, the *Sac*I-*Mun*I fragment of p12, the *Mun*I-*Nhe*I fragment of p11 and the *Nhe*I-*Xho*I fragment of p10 were ligated into *Sac*I-*Xho*I digested pRS2 plasmid. The resulting plasmid was named p17. The *Apa*I-*ApaL*I fragment of p15 and the *ApaL*I-*Xho*I fragment of p13 then were ligated into *Apa*I-*Xho*I treated pRS2, resulting in the plasmid named p18. Next, the *Apa*I-*Nsi*I fragment of p18 and the *Nsi*I-*Xho*I fragment of p17 were ligated together in *Apa*I-*Xho*I treated pRS2. The resulting

plasmid was named p19. Finally, the *Apa*I-*Avr*II fragment of p19, the *Avr*II-*Sal*GI fragment of p9 and the *Sal*GI-*Bam*HI fragment of p16 were ligated together into a previously constructed Sindbis replicon vector expressing the GFP reporter (see Dubensky et al., *J. Virol. 70*:508-519, 1996, Gardner et al., *J. Virol.*, 74:11849-11857, 2000 and U.S. Patent 5,843,723), that had also been treated with *Apa*I-*Bam*HI to remove the existing nonstructural protein genes. The resulting newly constructed replicon vector containing sequences derived from the SinDCchiron virus and expressing a GFP reporter was designated SINCR-GFP (also known as DCSP6SINgfp).

Similarly, the same Sindbis sequences were used for assembly into an alphavirus-based Eukaryotic Layered Vector Initiation System (see U.S. Patents 5,814,482 and 6,015,686, and Dubensky et al., 1996, *ibid*), in which the transcription of self-amplifying vector RNA takes place directly within eukaryotic cells via an RNA polymerase promoter (*e.g.*, CMV). An ELVIS plasmid DNA, which also expressed GFP reporter, was constructed by replacing Sindbis virus derived sequences in an existing ELVIS vector with the corresponding SINCR-GFP sequences from above. The previously described ELVIS vector pSIN1.5 (Hariharan et al., *J. Virol.* 72:950-958, 1998) first was modified by substituting the plasmid backbone with that from pCMVLink (zur Megede et al., *J. Virol.* 74:2628-2635, 2000) using two *Sac*I sites found in each plasmid, to generate the intermediate construct known as ELVIS1.5CB. Next, one of the two *Sac*I sites of ELVIS1.5CB (located adjacent to the SIN 3'-end) was eliminated by partially digesting with *Sac*I, blunt-ending using T4 DNA polymerase, and then ligating into the modified site, a *Pme*I linker 5'-GTTTAAAC-3'. The correct plasmid without the targeted *Sac*I site was designated ELVIS1.5CBdlSac. This intermediate plasmid then was prepared for insertion of the new SIN nonstructural protein genes by digestion with *Sac*I and *Xho*I. The corresponding nonstructural genes were obtained by PCR amplification from SINCR-GFP using the oligonucleotide primers

5'CCTATGAGCTCGTTTAGTGAACCGTATTGACGGCGTAGTACACAC (SEQ ID NO:) and 5'CCTATCTCGAGGGTGGTGTTGTAGTATTAGTC (SEQ ID NO:)

followed by digestion with *Sac*I and *Xho*I, and ligation, to produce the intermediate construct SINCP-Not. Finally, one of the two *Not*I sites present in this construct was eliminated by partial digest and Klenow fill-in, to leave only one *Not*I site in the polylinker. This newly constructed ELVIS vector was designated SINCP (or pSINCP).

Construction of a defective helper-based packaging cassette from clones of the SinDCchiron virus may be accomplished as follows. A *Bam*HI-*Sac*II fragment containing the Sindbis virus subgenomic promoter and 5' subgenomic NTR, from the previously described DH-BB helper plasmid (Bredenbeek et al., *J. Virol.* 67:6439-6446, 1993), a *Sac*II-*Nru*I fragment from clone p5 and a *Nru*I-*Hind*III fragment from p4 are cloned together into *Bam*HI-*Hind*III digested pRS2. The resulting plasmid is named p20. Next, the *Eco*RI-*Bsp*HI fragment of clone p3, the *Bsp*HI-*Spl*I fragment of clone p2 and the *Spl*I-*Nsi*I fragment of clone p1 are cloned into *Eco*R1-*Hind*III digested pRS2. The resulting plasmid is named p21. Finally, the *Bam*HI-*Bsu*36I fragment of p20 and the *Bsu*36I-*Nsi*I fragment of p21 are cloned into *Bam*HI-*Nsi*I digested DH-BB helper plasmid, resulting in the final packaging construct named DCSP6SINdh.

Other variations of defective helper based structural protein expression cassettes (packaging constructs) can be readily constructed. These include cassettes that express the alphavirus structural protein genes in a "split" configuration, as well as RNA polymerase II based constructs for use in the derivation of stable packaging cell lines (see U.S. Patents 5,789,245, 6,015,686, 6,015,694, 5,842,723, and 5,814,482, and WO 00/39318). For example, SP6-based defective helpers for *in vitro* transcription were constructed to contain only the envelope glycoprotein genes (but not a capsid protein gene) from the above-described Sindbis virus strains. Also included in these constructs was a Ross River virus translation enhancer element (WO 97/38087, WO 99/18226). Construction of such plasmids was performed stepwise as follows, using cDNA fragments from the DC+ virus (4.X.X, below) or LP virus (1.X.X, below).

The EcoRI-Bsu36 fragment from 4.4.2 and the Bsu36-HindIII fragment from 1.3.2 were cloned into EcoRI-HindIII digested pRS2 (designated cl201). The EcoRI-Bsu36 fragment from 4.4.2 and the Bsu36-HindIII fragment from 4.3.2 were cloned into

EcoRI-HindIII digested pRS2 (designated cl202). The EcoRI-SpII fragment from 4.2.2 and the SpII-HindIII fragment from 1.1.2 were cloned into EcoR1-HindIII digested pRS2 (designated cl203). The EcoRI-BspHI fragment from cl 201 and the BspHI-HindIII fragment from cl203 were cloned into EcoRI-HindIII digested pRS2 (designated cl221). The EcoRI-BspHI fragment from cl 202 and the BspHI-HindIII fragment from cl203 were cloned into EcoRI-HindIII digested pRS2 (cl222). The StuI-NsiI fragment of cl221 and the BspHI-StuI fragment of tRNABB/Cdel3rrv (Frolov et al, 1997, J. Virol. 71:2819-2829) were cloned into BspHI-NsiI digested tRNABB/Cdel3rrv (designated cl231). The StuI-NsiI fragment of cl222 and the BspHI-StuI fragment of tRNABB/Cdel3rrv were cloned into BspHI-NsiI digested tRNABB/Cdel3rrv (designated cl232).

Alphavirus vector replicon particles for use in evaluating human dendritic cell tropism, as well as for use *in vivo* (*e.g.*, vaccine administration), may be generated using a variety of previously described methodologies. These methods include, for example: 1) triple co-transfection of *in vitro* transcribed replicon vector and defective helper packaging RNAs, 2) co-transfection of plasmid DNA-based vector and packaging cassettes, and 3) introduction of vector replicon RNA into stable packaging cell lines (see for example, Dubensky et al., *J. Virol. 70*:508-519, 1996; Bredenbeek et al., *J. Virol. 67*:6439-6446, 1993; and U.S. Patents 5,843,723 and 5,789,245). The glycoprotein constructs (defective helpers) constructed above are used for packaging replicon particles by triple co-transfection of *in vitro* transcribed replicon, glycoprotein and capsid RNAs. In those instances where the glycoprotein defective helper clone 232 (tRNABB/Cdel3rrvDC) is used, corresponding vector replicon particles will efficiently infect human dendritic cells, particularly the immature population of DC. In those instances where the glycoprotein defective helper clone 231 (tRNABB/Cdel3rrvNDC) is used, corresponding replicon particles will not efficiently infect human dendritic cells. In addition, one additional glycoprotein expression cassette was constructed to include a three-nucleotide deletion that corresponds to E2 amino acid 160, in place of the amino acids present in the DC+ or LP versions (Gardner et al., *J. Virol.*, 74:11849, 2000). As demonstrated below, this amino acid deletion provides essentially the same human DC tropism observed with the E2 160

amino acid from the SinDCChiron virus isolate. Replicon particles of each configuration were produced for biological evaluation.

As shown if Figures 3, 4, 5 and 8 Sindbis-GFP replicon vector that was packaged in the structural proteins from SinDCchiron virus acquired the ability to efficiently infect primary human dendritic cells, particularly immature dendritic cells. In these experiments, FACS analysis was performed as follows. Developing dendritic cells were harvested from culture, washed in PBS/1%FCS and enumerated by trypan blue exclusion staining. Non-specific binding was blocked by incubation with human Ig, and cells were incubated with propidium iodide to enable subsequent analysis of viable cells. Cells (1-10x10^6) were stained at 4°C for 30 minutes with flourochrome-conjugated antibodies to dissect the cell population susceptible to infection. The following antibodies (and their cellular specificities) were used: CD1a-PE (pan-dendritic cell), CD86-PE (antigen presenting cells: dendritic cells, macrophages, B cells), CD83-PE (mature dendritic cells), HLA-ABC-PE (many cell types), HLA-DR-PE (antigen presenting cells: dendritic cells, macrophages, B cells), CD3 (T cells), CD5 (T cells and B cells), CD16 (NK cells), CD11c-PE (dendritic cells and macrophages) and CD14-PE (monocytes). All antibodies were obtained from Becton Dickinson. Isotype matched controls were used to establish quadrant positions. Analysis was performed on a FACScan (Becton Dickinson), and the data acquired to a Macintosh 7100 computer. CellQuest 3.1 software was used to analyze and display data.

We found that immature human dendritic cells differentiated from monocytes for 3-5 days with IL4 and GMCSF were highly susceptible to infection with the DC-tropic Sindbis vector particles expressing GFP. The phenotype of the susceptible cell population, based on cell surface markers was $CD1a^+CD83^-CD14^-CD3^-CD86^+HLA\text{-}DR^+HLA\text{-}ABC^+CD11c^+$. Adherent macrophages were differentiated from monocytes with MCSF (50U/ml; R&D Systems, Minneapolis, MN) for 6 days in 12-well Costar plates at 1.5 x 10^6 cells /ml. Adherent macrophages ($HLA\text{-}DR^+$) derived from these cultures were infected with particular Sindbis variant particles of the present invention. Macrophages developed in this system are known in the field to be closely related to dendritic cells, and can function as dendritic cell precursors. Purified $CD3^+$ T cells were not infected at equivalent MOIs, and a subset of dendritic cell precursors (HLA-

$DR^+CD14^{dim}CD3^-CD16^-CD1a^-CD5^-$) were infected when heterogeneous PBMC cultures were exposed to SIN replicon particles. Taken together, dendritic cells, and the macrophage and PBMC precursor populations were susceptible to infection with Sindbis-derived alphavirus particles of the present invention, and T cells, B cells and NK cells were refractory to infection.

When similar studies were performed with mouse derived dendritic cells, we demonstrated that packaging of alphavirus replicon particles with the structural proteins from wild-type alphaviruses (*e.g.*, Sindbis virus, Semliki Forest virus) could provide for efficient vector particle infection of mouse DC, but not human DC (see Figure 8, SFV or SIN LP). Only by using modified or adapted structural proteins can these vector particles efficiently infect human dendritic cells (see SIN DC+).

Infection of human dendritic cells was receptor mediated and could be inhibited by an anti-virion rabbit polyclonal antibody. For example, GFP-expressing replicon particles packaged with the SinDCchiron structural proteins (2×10^7) were pre-incubated for 30 minutes with either anti-virion antibody or control rabbit polyclonal antibody recognizing mouse Ig (from Sigma, Cat # M-6024) at 1:100 dilution in PBS. Immature human dendritic cells (2×10^5; Day 3) were infected for 1 hour and analyzed 24 hours later for GFP expression. Anti-virion antibody blocked infection of dendritic cells by SinDCchiron particles, whereas non-specific antibody had no effect.

We next wished to determine whether alphavirus vector particles selected for human DC-tropism could be used as a method to induce maturation and activation in dendritic cells that have been transduced with the vector, either in vitro or in vivo. As shown in Figure 9, human DC transduced in vitro with SIN-GFP vector particles, show significant up-regulation of the cell surface markers CD80, CD86, CD83, and MHC class II, which are known markers for maturation and activation. In addition, similar results were observed in vivo, for a mouse model. Following peripheral inoculation with SIN-GFP vector particles, the CD11c dendritic cells positive for GFP expression, were isolated from the draining lymph nodes and shown to have significant up-regulation of maturation and activation markers (*e.g.*, MHC II).

To further demonstrate the ability of DC-tropic Sindbis-GFP particles to infect dendritic cells in vivo, we inoculated groups of 4 female Balb/C mice aged 8-10 weeks intradermally in the ear. The left ear was painted with 25ul rhodamine B isothiocyanate (2% dissolved in acetone/dibutylpthalate) prior to injection with 25ul SIN-GFP particles (1.75×10^7 total particles in formulation buffer). The right ear was not painted with rhodmaine and was injected with SIN-GFP particles (1.75×10^7) alone. Control groups received formulation buffer alone, and the left ear of each animal was painted with rhodamine. Two animals from each group were anesthetized and exsanguinated at 24 hours and 48 hours post injection, and the ears and draining mandibular node were harvested immediately into1% paraformaldehyde. The tissues were fixed for 72 hours at 4°C in the dark, before embedding in paraffin. Tissue sections (5um) were prepared with a cryostat onto glass microscope slides and analyzed quantitatively with a laser scanning cytometer (Compucyte, Cambridge, MA), or photo-documented with a fluorescent microscope (Zeiss) linked to a CCD camera (Figures 6 and 7). The presence of cells with GFP and rhodamine in the draining lymph nodes provides strong evidence that migratory dendritic cells are infected with the Sindbis replicon particles in the skin, and traffic to the node. The cells exhibit dendritic morphology and have numerous processes that contact neighboring cells in the node. No GFP expressing cells were detected in the control groups.

To demonstrate the utility of human DC-adapted alphavirus vectors of the present invention as a "tool" or method for performing in vitro assays of antigen presentation and stimulation of immune cells (*e.g.*, class I restricted T cell responses), we utilized as a representative example, a murine T cell hybridoma assay (Figure 10). The murine T cell hybridoma 12.2 was generated by fusion of splenocytes from HIV-gag-immunized CB6F1 mice with the BWZ.36 fusion partner, followed by cloning and selection based on IL-2 production in response to APCs loaded with HIV-derived peptides. The 12.2 T cell hybridoma specifically recognizes the peptide sequence AMQMLKETI (p7g) of the HIV-1 SF2 p24gag protein in the context of H-2Kd. The 12.2 T hybridoma produces IL-2 in a dose-responsive manner upon co-culture with p7g peptide-loaded H-2Kd DC and is unresponsive to DC loaded with another gag-derived peptide SQVTNPANI (gagb). The p7g gag-specific T cell hybridoma 12.2 was plated

at 10^6 cells/ml, 10^5 cells/well in 96-well, U-bottom microtiter plates. Varying numbers of DC were added to wells for a total volume of 200µl. As a positive control for DC function, DC from each condition were assayed in the presence of 1 ng/ml p7g peptide, as well as in media alone. Negative control wells containing DC or the T cell hybridoma alone were also included in each experiment and reliably yielded <20 pg/ml IL2. Each experimental condition was assayed in duplicate. After 24 hr co-culture at 37°C, supernatants were removed and assayed for IL-2 production by ELISA according to manufacturer's instructions (Endogen, Woburn, MA).

As a permanent source of homogeneous dendritic cell adapted virus, full-length cDNA clones, from which infectious SinDCchiron virus genomic RNA may be transcribed in vitro, may be constructed by using the SINCR-GFP (DCSP6SINgfp) vector as starting material. Specifically, the *Apa*I-*Sal*GI and *Sal*GI-*Bam*HI fragments from DCSP6SINgfp vector, plus the *Bam*HI-*Xho*I from the DCSP6SINdh packaging construct are cloned into pRS2 that has been digested with *Apa*I and *Xho*I. The resulting construct is designated DCSP6SINgen.

Alternatively, full-length cDNA clones of the SinDCchiron and SinChironLP viruses, as well as another version of Sindbis virus containing the E2 160 deletion (designated SinDCdl160), may be constructed similarly as follows. A cDNA fragment comprising the subgenomic promoter and capsid protein gene was re-amplified by RT-PCR from virus genome RNA using specific oligonucleotides that will result in a fragment of sufficient length to include the *BamH*I and *Stu*I restriction sites. This PCR amplicon was digested with *BamH*I and *Stu*I, and ligated with *Stu*I and *Nsi*I digested envelope glycoprotein genes from each of the glycoprotein cassettes (DC+, LP, dl160) described above, and also plasmid pRS2 that had been digested with *BamH*I and *Nsi*I. The three resulting constructs, designated pRS2spLP, pRS2spDC+, and pRS2spdl160 were next digested with *BamH*I and *Nsi*I to isolate the structural protein gene fragment and these fragments were then ligated with the *Pme*I-*BamH*I fragment (backbone and partial nonstructural protein genes) and *Nsi*I-*Pme*I fragment (3'-end) from SINCR-GFP. Finally, the *BamH*I-*BamH*I fragment from SINCR-GFP (remaining nonstructural protein genes) was inserted into the above three constructs that had been digested with *BamH*I, resulting in three different full-length Sindbis cDNA clones

(SINDC+gen, SINLPgen, SINdl160gen) that differed only by the amino acid (or deletion) at E2 residue 160.

Infectious virus seed stocks were generated from these plasmids by linearization of the plasmids with *Pme*I, genome RNA transcription *in vitro*, and transfection of BHK-21 cells using methods documented in previous publications (see for example Rice et al., *J. Virol. 61*:3809-3819, 1987; Dubensky et al., *J. Virol. 70*:508-519, 1996; Bredenbeek et al., *J. Virol. 67*:6439-6446, 1993; and U.S. Patent 5,843,723). Culture supernatants containing infectious virus were harvested, clarified and titered by standard plaque assay using BHK-21 cells.

Alphavirus packaging cell lines of the present invention, containing Sindbis glycoproteins that provide for human DC tropism, were constructed using approaches that we have described previously (U.S. Patents 5789245, 5843723 and 6015694, PCT publications WO 97/38087 and WO 99/18226, and Polo et al., *PNAS*, 96:4598, 1999). Specifically, separate glycoprotein and capsid structural gene expression cassettes were constructed, such that each contained a selectable drug resistance marker (neomycin^r or hygromycin^r, respectively). The glycoprotein and capsid protein genes were amplified from the above cDNAs using 5'- and 3'-end specific oligonucleotide primers and inserted into the structural gene cassette backbones. The resulting glycoprotein constructs were designated pE3n-LP and pE3n-DC+, and the capsid construct pE3h-Cap.

To generate stable alphavirus packaging cell lines, plasmid pE3h-Cap was first transfected into cells (BHK cell, as an example) using the calcium phosphate precipitation method. Hygromycin resistant cells were selected following the addition of drug (300 ug/ml). Subsequently, these hygromycin resistant cells were transfected with a glycoprotein expression plasmid (pE3n-LP and pE3n-DC+, independently) and the cells then were placed under neomycin (G418, 500-600 ug/ml) drug selection. Resistant cells were obtained and cloned by limiting dilution.

Identification of drug resistant cell lines that exhibited high level packaging activity was performed initially by transfection of the cells with a plasmid DNA-based Sindbis replicon vector expressing β-galactosidase (pSIN1.5bgal, Hariharan et al., J. Virol.,

72:950, 1998), harvesting culture supernatants approximately 24 hr post-transfection, and assaying the supernatants for the presence of packaged SIN replicon particles by infection of naïve BHK cells. Packaging cell lines derived from both the human DC-tropic and the LP glycoproteins were obtained. Those with highest packaging activity were then compared to quantitate both the level and duration of replicon packaging activity (see Figure 13). Packaging cell lines were infected at low multiplicity (MOI=0.5) with a seed stock of SIN replicon particles expressing β-galactosidase. Complete media exchanges were performed at the indicated time points and the harvested culture supernatants were assayed for the level of packaged SIN replicon particles by infection of naïve BHK cells. As shown in Figure 13, these cell lines were able to package replicon particles at levels ranging from 10^8 to 10^9 IU/mL, across a number of harvests. In addition, the supernatants containing packaged SIN replicon particles were shown to be free from contaminating replication-competent virus by the methods of blind serial passage in naïve BHK cells, as well as standard plaque assay.

Example 3

Prime-Boost Vaccine Strategies for Alphavirus Vectors

In order to be optimally efficacious, a vaccine for a given pathogenic agent (*e.g.*, infectious disease agents such as viruses, bacteria, fungi, parasites) must stimulate a robust and broad-based antigen-specific immune response. The vaccinated individual will be resistant to development of sequelae characteristic of the infectious organism upon subsequent challenge, as a result of stimulation of residing memory cells and maturation into antigen-specific effector cells, which facilitate clearing of the offending infectious agent. Similarly, a therapeutic vaccine for a given infectious disease must also stimulate a robust and broad-based antigen-specific immune response, to clear or to diminish the extent of infectious disease.

Generally, vaccines are given in greater than one, and often in many, doses. The rationale of such an immunization strategy is to "boost" the "prime" response, resulting in a more durable immune response that is characterized by an increased capacity of an individual to resist challenge of an infectious organism. This increased resistance to challenge is due to a larger number of residing immune memory cells, often with

specificity for a broader range of antigenic epitopes, corresponding to the infectious organism.

A broad-based antigen-specific immune response results when vaccination elicits both T-helper cell 1 (Th1) and T-helper cell 2 (Th2) responses. Proliferation of Th1 and Th2 cells are distinguished by their pattern of cytokine secretion, the Th1 subset corresponding to interleukin 2 (IL-2), and IFN-γ, and the Th2 subset corresponding to IL-4, IL-5, IL-6, and IL-10. Antigens expressed as a result of vaccination are presented to the immune system via dendritic cells (DCs), so-called "professional antigen presenting cells." DCs initiate and modulate the immune response. In particular, DCs are potent stimulators of B and T lymphocytes (for review, see Banchereau and Steinman, *Nature* 392:245-252, 1998). Stimulation of the T and B lymphocyte antigen-specific effectors occurs by presentation by the dendritic antigen presentation cells, in which processed antigen is displayed in conjunction with major histocompatibility complex (MHC) molecules of two alternative types (MHC class I, or MHC class II), to T lymphocytes, via the T-cell antigen receptor. Antigen presentation via MHC class I results in a cellular CD8+ cell (cytotoxic T cell, CTL) response, whereas antigen presentation via MHC class II results in stimulation of CD4+ cells, and subsequently B lymphocytes, resulting in a humoral (antibody, Ab) response. Thus, the rationale of a "prime"-"boost" vaccination strategy is to elicit a broad and durable Th1 and Th2 antigen-specific immune response.

Traditional vaccination prime-boost strategies have used only a single modality for both the prime and boost steps, and may not, for particular infectious diseases, elicit a broad Th1/Th2 antigen-specific immune response. Thus, within the scope of the prevention invention, several "mixed" modalities of prime-boost regimes are disclosed wherein a variety of vaccinating compositions may be administered either as a "prime" or "boost", in conjunction with an alphavirus replicon vector (*e.g.*, as recombinant particles, DNA or RNA) encoding a designated antigen. Such a prime-boost immunization strategy incorporating alphavirus vector replicons will elicit a much more durable and broad-based Th1/Th2 antigen-specific immune response in the vaccinated individual. The combined prime-boost immunization modalities may include, for example, plasmid DNA (formulated or non-formulated), ELVIS vector,

recombinant alphavirus particles, other non-alphaviral vectors and recombinant protein. Any of these vaccine modalities, including the alphavirus vector replicons encoding a designated antigen, may be given with an adjuvant. Representative examples of adjuvants that can be utilized are MF59, poly-D-galactoside, alum, CpG oligonucleotides, PLG microparticles or mono-phosphoro lipid.

To demonstrate the utility of prime-boost regimes according to the present invention, HIV gag and envelope antigens were chosen as an example. HIV gag and HIV gp140env were expressed by plasmid DNA vectors (*e.g.*, conventional CMV promoter vectors), ELVIS vector and alphavirus vector particles, and also produced as a recombinant protein. Other antigens from HIV, as well as antigens from other pathogenic agents (for example HCV), similarly may be used by one of skill in the art based on the teachings of the present invention. In addition, a variety of widely available non-alphavirus viral vectors (*e.g.*, poxvirus) may be used to express any of the aforementioned antigens.

A. Construction of HIV gag and envelope expression vectors

The HIV gag coding sequence was selected from the HIV-1SF2 strain (Sanchez-Pescador, R., et al., *Science* 227(4686):484-492, 1985; Luciw, P.A., et al., U.S. Patent No. 5,156,949, issued October 20, 1992, herein incorporated by reference; Luciw, P.A., et al., U.S. Patent No. 5,688,688, November 18, 1997). These sequences have been used directly or first manipulated to maximize expression of their gene products. For maximization of expression, the HIV-1 codon usage pattern was modified so that the resulting nucleic acid coding sequence was comparable to codon usage found in highly expressed human genes. The HIV codon usage reflects a high content of the nucleotides A or T as third base of the codon-triplet. The effect of the HIV-1 codon usage is a high AT content in the DNA sequence that could result in a decreased translation ability and instability of the mRNA. In comparison, highly expressed human codons prefer the nucleotides G or C as the third base. The gag coding sequence therefore was modified to be comparable to codon usage found in highly expressed human genes (zur Megede, *J. Virol.* 74:2628, 2000).

The DNA fragment for gag was cloned into the eukaryotic expression vector pCMVKm2, derived from pCMV6a (Chapman et al., *Nuc. Acids Res. 19*:3979-3986, 1991) and comprising a kanamycin selectable marker, a ColE1 origin of replication, a CMV promoter enhancer and Intron A, followed by cloning sites for insertion of sequences, followed by a polyadenylation signal derived from bovine growth hormone. The gag sequence-containing vector was designated pCMVKm2.GagMod.SF2. This plasmid was deposited January 18, 1999, with the Chiron Corporation Master Culture Collection, Emeryville, CA, 94662-8097, and with the American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209.

The DNA fragment encoding HIV gag was then cloned into an alphavirus plasmid DNA vector (ELVIS), and replicon vectors (SINBV and SINCR) to be used for the generation of recombinant alphavirus particles. Specifically, a construct for *in vitro* transcription of Sindbis virus RNA vector replicons (pRSIN-luc; Dubensky et al., *J Virol.* 10:508-519, 1996) was modified to contain a *Pme*I site for plasmid linearization and a polylinker for insertion of heterologous genes. First, a polylinker was generated using two oligonucleotides that contain the sites *Xho*I, *Pml*I, *Apa*I, *Nar*I, *Xba*I, *and Not*I.

XPANXNF: 5'GCA CGT GGG CCC GGC GCC TCT AGA GC

XPANXNR: 5'GCT CTA GAG GCG CCG GGC CCA CGT GC

The plasmid pRSIN-luc (Dubensky et al., *supra)* then was digested with *Xho*I and *Not*I to remove the luciferase gene insert, blunt-ended using Klenow and dNTPs, and ligated with the oligonucleotides that were annealed to each other. The resulting construct was digested with *Not*I and *Sac*I to remove the minimal Sindbis 3'-end sequence and A_{40} tract, and ligated with an approximately 0.4 kbp fragment from pKSSIN1-BV (WO 97/38087), obtained by digestion with *Not*I and *Sac*I. The fragment contained the complete Sindbis virus 3'-end, an A_{40} tract and a *Pme*I site for linearization. This replicon vector construct was designated SINBVE. The other replicon vector, SINCR, was described previously in example 2.

The HIV gag coding sequence was obtained from the parental pCMVKm2.GagMod.SF2 plasmid by digestion with *EcoR*I, blunt-ending with Klenow and dNTPs, purification with GeneCleanII, and digestion with *Sal*I. The HIV gag-coding fragment then was ligated into the SINBVE vector that had been digested with *Xho*I and *Pml*I. The resulting vector was designated SINBV-gag. In parallel, the same HIV gag fragment was inserted into the new replicon of the present invention, by ligating with SINCR-GFP vector that had been digested with *Not*I and blunt-ended, followed by digestion with *Xho*I, to remove the GFP reporter gene insert. This vector was designated SINCR-gag. Vector RNA replicons were packaged into recombinant alphavirus particles by using previously described methods (see for example Dubensky, et al., *J Virol. 70*:508-519, 1996; Polo et al., *PNAS* 96:4598-4603; and U.S. Patents 5,789,245, 5,842,723, and 6,015,694, which are incorporated by reference in their entirety).

The construction of a new SIN vector replicon utilizing the nonstructural gene sequences obtained from SinDCchiron virus (ATCC# VR-2643) or SinChironLP virus, described in Figures 2B and 2C, resulted in a replicon that had superior properties as compared to a replicon derived from wild-type SIN virus (*e.g.*, ATCC# VR-2526). These properties include, but are not limited to, enhanced expression in human dendritic cells, increased packaging into recombinant alphavirus particles at levels as much as 10-fold over wild-type replicons, and the ability to induce a more robust immune response following immunization of animals. For example, Figure 11 compares the induction of HIV gag specific CD8 T cells in mice following immunization with DC-tropic vector particles containing either the new SIN replicon (SINCR) or a previously described wild-type SIN replicon (SINBV). At doses of either 10^5 or 10^7 IU, the SINCR replicon that contains nonstructural gene sequences from SinDCchiron virus and SinChironLP virus is clearly more potent at immune induction.

For construction of an alphavirus DNA-based vector (ELVIS) expressing HIV gag, plasmid pDCMVSIN-β-gal (Dubensky et al., *J Virol. 70*:508-519, 1996) was digested with *Sal*I and *Xba*I, to remove the beta-galactosidase gene insert, and the HIV gag gene was inserted after digestion and purification of the fragment from SINBV-gag. The resulting construct was designated pDSIN-Gag. In addition, the same HIV gag

insert was inserted into the ELVIS replicon of the present invention (SINCP, Example 2 above). Similarly, constructs expressing HIV envelope (env) protein were made using the same replicon and ELVIS vector backbones. A variety of envelope sequences have been used for these constructions and the parental plasmids used for isolation of the env genes (*e.g.*, pCMVgp160.modUS4, pCMVgp140.mut7.modSF162, pCMVgp140.mut8.modSF162) have been deposited with CMCC and ATCC and described previously (WO 00/39302).

For prime-studies incorporating recombinant proteins, recombinant HIV gag protein was obtained using a baculovirus expression system. A baculovirus shuttle vector containing the synthetic HIV gag sequence was constructed as follows. The synthetic HIV p55 gag expression cassette (described above) was digested with restriction enzyme *Sal*I followed by incubation with T4-DNA polymerase. The resulting fragment was isolated and then digested with *BamHI*. The shuttle vector pAcCl3 (Munemitsu S., et al., *Mol Cell Biol.* 10(11):5977-5982, 1990) was linearized by digestion with *EcoRV*, followed by incubation *with T4-DNA polymerase. The linearized vector was digested with *Bam*HI, treated with alkaline phosphatase, and the fragment isolated from an agarose gel. The isolated 1.5 kb fragment was ligated with the prepared pAcC13 vector, resulting in the clone designated pAcC13-Modif.p55gag.

Generation of the recombinant baculovirus was achieved by co-transfecting 2 µg of the HIV p55 gag shuttle vector with 0.5 µg of linearized, *Autographa californica* baculovirus (AcNPV) wild-type viral DNA into *Spodoptera frugiperda* (Sf9) cells (Kitts, P.A., Ayres M.D., and Possee R.D., *Nucleic Acids Res.* 18:5667-5672, 1990). The isolation of recombinant virus expressing HIV p55 Gag was performed according to standard techniques (O'Reilly, D.R., L.K. Miller, and V. A. Luckow, *Baculovirus Expression Vector: A Laboratory Manual*, W.H. Freeman and Company, New York, 1992).

Expression of the HIV p55 Gag was achieved using a 500 ml suspension culture of Sf9 cells grown in serum-free medium (Maiorella, et al, *Bio/Technology* **6**:1506-1510, 1988) that had been infected with the HIV p55 Gag recombinant baculovirus at a multiplicity of infection (MOI) of 10. Forty-eight hours post-infection, the supernatant

was separated by centrifugation and filtered through a 0.2 □m filter. Aliquots of the supernatant were then transferred to Polyclear™ (Beckman Instruments, Palo Alto, CA) ultracentrifuge tubes, underlaid with 20% (wt/wt) sucrose, and subjected to 2 hours centrifugation at 24,00 rpm using a Beckman SW28 rotor.

The resulting pellet was suspended in Tris buffer (20 mM Tris HCl, pH 7.5, 250 mM NaCl, and 2.5 mM EDTA), layered onto a 20-60% (wt/wt) sucrose gradient, and subjected to 2 hours centrifugation at 40,000 rpm using a Beckman SW41*ti* rotor. The gradient was then fractionated starting at the top (20% sucrose) of the gradient into approximately twelve 0.75 ml aliquots. A sample of each fraction was electrophoresed on 8-16% SDS polyacrylamide gels and the resulting bands were visualized after commassie staining. Additional aliquots were subjected to refractive index analysis.

The results indicated that the p55 gag VLP banded at a sucrose density of range of 1.15 - 1.19 g/ml with the peak at approximately 1.17 g/ml. The peak fractions were pooled and concentrated by a second 20% sucrose pelleting. The resulting pellet was suspended in 1 ml of Tris buffer (described above). The total protein yield as estimated by Bicimchrominic Acid (BCA) (Pierce Chemical, Rockford, IL). Other forms of HIV gag, for example that which has been expressed in recombinant form, but not as VLPs may similarly be used (WO 00/39302).

Recombinant HIV envelope proteins were expressed from stable mammalian cell lines, rather than from baculovirus systems. Chinese hamster ovary (CHO) cells were transfected with plasmid DNA encoding the synthetic HIV-1 env proteins using Mirus TransIT-LT1 polyamine transfection reagent (Pan Vera) according to the manufacturers instructions and incubated for 96 hours. After 96 hours, media was changed to selective media (F12 special with 250 µg/ml G418) and cells were split 1:5 and incubated for an additional 48 hours. Media was changed every 5-7 days until colonies started forming at which time the colonies were picked, plated into 96 well plates and screened by gp120 Capture ELISA. Positive clones were expanded in 24 well plates and screened several times for Env protein production by Capture ELISA, as described above. After reaching confluency in 24 well plates, positive clones were

expanded to T25 flasks (Corning, Corning, NY). These were screened several times after confluency and positive clones were expanded to T75 flasks.

Positive T75 clones were frozen in LN2 and the highest expressing clones amplified with 0-5 μM methotrexate (MTX) at several concentrations and plated in 100mm culture dishes. Plates were screened for colony formation and all positive closed were again expanded as described above. Clones were expanded an amplified and screened at each step by gp120 capture ELISA. Positive clones were frozen at each methotrexate level. Highest producing clones were grown in perfusion bioreactors (3L, 100L) for expansion and adaptation to low serum suspension culture conditions for scale-up to larger bioreactors.

The following table shows capture ELISA data from CHO cells stably transfected with plasmid vectors carrying a cassette encoding synthetic HIV-SF162 Env polypeptides (*e.g.*, mutated cleavage sites, modified codon usage and/or deleted hypervariable regions). Thus, stably transfected CHO cell lines which express Env polypeptides (*e.g.*, gp120, gp140-monomeric, and gp140-oligomeric) have been produced.

Table 2

CHO Cell Lines Expression Level of SF162 Envelope Constructs			
Constructs	CHO Clone #	MTX Level	Expression Level* (ng/ml)
gp120.modSF162	1	0	755-2705
	2	0	928-1538
	3	0	538-1609
gp140.modSF162	1	20 nM	180-350
gp140.mut. modSF162	1	20 nM	164-451
	2	20 nM	188-487

CHO Cell Lines Expression Level of SF162 Envelope Constructs			
Constructs	CHO Clone #	MTX Level	Expression Level* (ng/ml)
	3	20 nM	233-804
gp120.modSF162.delV2	1	800nM	528-1560
	2	800nM	487-1878
	3	800nM	589-1212
gp140.modSF162.delV2	1	800nM	300-600
	2	800nM	200-400
	3	800nM	200-500
gp140.mut.modSF162.delV2	1	800nM	300-700
	2	400nM	1161
	3	800nM	400-600
	4	400nM	1600-2176

*All samples measured at T-75 flask stage unless otherwise indicated

The results presented above demonstrate the ability of the constructs of the present invention to provide expression of Env polypeptides in CHO cells. Production of polypeptides using CHO cells provides (i) correct glycosylation patterns and protein conformation (as determined by binding to panel of MAbs); (ii) correct binding to CD4 receptor molecules; (iii) absence of non-mammalian cell contaminants (*e.g.*, insect viruses and/or cells); and (iv) ease of purification.

In certain embodiments, it may be desirable to formulate plasmid DNA preparations (*e.g.*, ELVIS vectors) using PLG/CTAB microparticles (Singh et al, *PNAS*, 97:811,

2000). Specifically, antigen-encoding alphavirus-based ELVIS plasmids of the present invention were formulated with microparticles as briefly outlined below.

First, blank microparticles were produced using 4% RG 504 PLG (Boehringer Ingelheim) in dimethyl chloride and 0.5% CTAB (Sigma Chemical Co., St. Louis, MO) in water. In particular, the microparticles were made by combining 12.5 ml of polymer solution with 1.25 ml of distilled water and homogenizing for 3 minutes using an Omni benchtop homogenizer with a 10 mm probe at 10K rpm to form a w/o emulsion. The w/o emulsion was added to 50 ml of the 0.5% CTAB solution and homogenized for 3 minutes to form a w/o/w emulsion. The w/o/w emulsion was left stirring overnight for solvent evaporation, forming microparticles. The formed microparticles were then filtered through a 38 □ mesh, washed with water by centrifugation 4 times, and lyophilized. The microparticles were then sized in a Malvern Master sizer for future use. Next, the microparticles adsorbed with ELVIS plasmid DNA were prepared by incubating 20 mg of blank microparticles with increasing concentrations of plasmid DNA in a 1.0 ml volume for 3 hours at 4^0C. Following incubation, the microparticles were centrifuged, washed twice with Tris-EDTA buffer and freeze-dried overnight.

B. Demonstration of enhancement by prime-boost vaccination regimes

To demonstrate the ability to improve the immunogenicity of vaccines using a prime-boost regime that takes advantage of alphavirus vectors, a variety of different combinations may be used. For example, in one embodiment, alphavirus vector particles may be administered as a prime followed by a recombinant protein, DNA vector or non-alphavirus (*e.g.*, poxvirus) viral vector boost. Alternatively, in another embodiment, a plasmid DNA vector or ELVIS vector may be administered as a prime followed by an alphavirus vector particle boost. Further, in another embodiment, an ELVIS vector prime may be administered with a recombinant protein or non-alphavirus (*e.g.*, poxvirus) viral vector boost. In yet another embodiment, alphavirus vector particles derived from a first alphavirus may be administered as a prime followed by alphavirus vector particles from a second alphavirus as a boost. It should be evident from the disclosure provided herein that a large number of different combinations of the above prime-boost elements may be interchanged, and that vectors (alphaviral or

non-alphaviral) encoding a variety of antigens may be similarly administered, using a variety of immunization routes.

In one example, to illustrate the benefits of such prime-boost approaches, animal immunization studies were performed using the SINBV-gag vector particles and pCMVKm2.GagMod.SF2 plasmid from above. These studies utilized a highly quantitative vaccinia virus challenge model to clearly elucidate CTL response differences in immunized animals. Groups of three mice per sample were immunized intramuscularly with DNA or alphavirus vector particles, in a 100 ul volume. Four weeks later, the mice were boosted with SIN vector particles (same or opposite vaccine compositions as initial priming immunization). Four weeks after boosting, the animals were then challenged intraperitoneally with 10^7 PFU of recombinant vaccinia virus expressing HIV gag. Five days later, direct spleen cell CTL assays were performed against gag peptide-pulsed, ^{51}Cr-labeled target cells. As shown in Figure 12, a DNA prime - alphavirus vector particle boost regime resulted in greater immune induction than DNA alone or vector particles alone.

In a second prime-boost example, animal immunization studies were performed in primates using an alphavirus-based ELVIS vector (SINCP) as prime and a recombinant protein as boost. Specifically, macaques (5 per group) were immunized with 1.0 mg of PLG formulated SINCP plasmid encoding HIV gp140 as a prime (3 times at 0, 4, and 14 weeks) and subsequently with recombinant oligomeric HIV gp140dV2 protein in MF59 adjuvant as a boost (1 time at 38 weeks). Examination of envelope-specific cellular and humoral immune responses was conducted at regular intervals, including those shown in Figure (14). Both lymphoproliferative (LPA) and antibody responses shown in Figure (14) were clearly evident after ELVIS DNA priming, with a peak at 11 weeks. As expected, these responses gradually decreased over time leading up to the week preceding the protein boost (week 37). Following immunization with the protein component at week 38, significant boosting of both the LPA and antibody responses to high levels were observed (week 40).

It should be appreciated, that the order of vaccine components delivered (*e.g.*, alphavirus vector particles, DNA, ELVIS, recombinant protein, non-alphavirus viral

vector), as well as the timing, dose and route of immunization, may be readily varied by one of skill in the art to include numerous possible combinations of the above. For example, the interval between prime and boost immunizations may range from slightly more than no interval (administration on the same day), to one or more days of interval, preferably at least 14 days, and more preferably at least 28 days and most preferably at least 2, 3, 4, 5, or 6 months. In addition, the route of administration of the various prime-boost elements is not limited to intramuscular delivery; rather, other routes (for example, intradermal, intravenous, subcutaneous, intranasal, and oral) also may be utilized and the priming route may differ from the boosting route.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

What is claimed is:

1. An immunogenic composition comprising:

a first immunizing component selected from the group consisting of: native antigens, recombinant antigens, non-alphavirus viral vectors, nucleic acids, and alphavirus derived vector systems selected from the group consisting of alphavirus vector particles, eukaryotic layered vector initiation systems, and RNA vector replicons; and

a second immunizing component selected from the group consisting of: native antigens, recombinant antigens, non-alphavirus viral vectors, nucleic acids, and alphavirus derived vector systems selected from the group consisting of alphavirus vector particles, eukaryotic layered vector initiation systems, and RNA vector replicons, with the proviso that said first immunizing component is different from said second immunizing component and that said first immunizing component, said second immunizing component or both are alphavirus derived vector systems.

2. An immunogenic composition comprising:

a first immunizing component comprising an alphavirus derived vector system selected from the group consisting of: alphavirus vector particles, eukaryotic layered vector initiation systems, and RNA vector replicons; and

a second immunizing component selected from the group consisting of native antigens, recombinant antigens, non-alphavirus viral vectors, nucleic acids, and alphavirus derived vector systems selected from the group consisting of alphavirus vector particles, eukaryotic layered vector initiation systems, and RNA vector replicons, with the proviso that said first immunizing component is different from said second immunizing component.

3. An immunogenic composition comprising:

a first immunizing component selected from the group consisting of native antigens, recombinant antigens, non-alphavirus viral vectors, nucleic acids, and

alphavirus derived vector systems selected from the group consisting of alphavirus vector particles, eukaryotic layered vector initiation systems, and RNA vector replicons; and

a second immunizing component selected from the group consisting of alphavirus derived vector systems selected from the group consisting of alphavirus vector particles, eukaryotic layered vector initiation systems, and RNA vector replicons, with the proviso that said first immunizing component is different from said second immunizing component.

4. The immunogenic composition according to claims 1, 2, or 3 wherein said first immunizing component, said second immunizing component, or both further comprise an adjuvant.

5. The immunogenic composition according to claim 4 wherein said adjuvant is selected from the group consisting of; aluminum salts, oil-in-water, saponin, cytokines, and oligonucleotides or polymeric molecules encoding immunostimulatory CpG motifs.

6. The immunogenic composition according to claim 5 wherein said aluminum salts are selected from the group consisting of aluminum hydroxide, aluminum phosphate, and aluminum sulfate.

7. The immunogenic composition according to claim 5 wherein said oil-in-water adjuvants are selected from the group consisting of MF59, SAF, Ribi™, complete Freunds adjuvant, and incomplete Freunds adjuvant.

8. The immunogenic composition according to claims 1, 2, or 3 wherein said native antigens are selected from the group consisting of polypeptides, proteins, polysaccharides, carbohydrates, nucleic acids and conjugates thereof.

9. The immunogenic composition according to claim 8 wherein said native antigens are derived from viruses, bacteria, fungi, or protozoans.

10. The immunogenic composition according to claims 1, 2, or 3 wherein said recombinant antigens are selected from the group consisting of polypeptides, proteins, polysaccharides, carbohydrates, nucleic acids and conjugates thereof.

11. The immunogenic composition according to claim 10 wherein said recombinant antigens are derived from viruses, bacteria, fungi, cancerous cells or protozoans.

12. The immunogenic composition according to claims 1, 2, or 3 wherein said immunogenic composition induces in its recipient a immune response that prevents, palliates or treats a disease.

13. The immunogenic composition according to claims 1, 2, or 3 wherein said immunogenic composition is a vaccine.

14. The immunogenic composition according to claim 12 wherein said recipient is an animal.

15. The immunogenic composition according to claim 14 wherein said animal is a human.

16. A method for inducing an immune response in an animal comprising:

administering a first immunizing component selected from the group consisting of: native antigens, recombinant antigens, non-alphavirus viral vectors, nucleic acids, and alphavirus derived vector systems selected from the group consisting of alphavirus vector particles, eukaryotic layered vector initiation systems, and RNA vector replicons; and

administering a second immunizing component selected from the group consisting of: native antigens, recombinant antigens, non-alphavirus viral vectors, nucleic acids, and alphavirus derived vector systems selected from the group consisting of alphavirus vector particles, eukaryotic layered vector initiation systems, and RNA vector replicons, with the proviso that said first immunizing component is different from said second immunizing component and that said first immunizing

component, said second immunizing component or both are alphavirus derived vector systems.

17. A method for inducing an immune response in an animal comprising:

administering a first immunizing component comprising an alphavirus derived vector system selected from the group consisting of: alphavirus vector particles, eukaryotic layered vector initiation systems, and RNA vector replicons; and

administering a second immunizing component selected from the group consisting of native antigens, recombinant antigens, non-alphavirus viral vectors, nucleic acids, and alphavirus derived vector systems selected from the group consisting of alphavirus vector particles, eukaryotic layered vector initiation systems, and RNA vector replicons, with the proviso that said first immunizing component is different from said second immunizing component.

18. A method for inducing an immune response in an animal comprising:

administering a first immunizing component selected from the group consisting of native antigens, recombinant antigens, non-alphavirus viral vectors, nucleic acids, and alphavirus derived vector systems selected from the group consisting of alphavirus vector particles, eukaryotic layered vector initiation systems, and RNA vector replicons; and

administering a second immunizing component selected from the group consisting of alphavirus derived vector systems selected from the group consisting of alphavirus vector particles, eukaryotic layered vector initiation systems, and RNA vector replicons, with the proviso that said first immunizing component is different from said second immunizing component.

19. A method for inducing an immune response in an animal according to claims 16, 17, or 18 further comprising administering said first immunogenic component multiple times.

20. A method for inducing an immune response in an animal according to claims 16, 17, or 18 further comprising administering said second immunogenic component multiple times.

21. A method for inducing an immune response in an animal according to claims 16, 17, or 18 wherein said first and said second immunogenic compositions are administered using a method selected the group consisting of intraocularly, intranasally, sublinqually, orally, topically, intravesically, intrathecally, intravaginally, intrarectally, intravenously, intraperitoneally, intracranially, intramuscularly, intradermally, and subcutaneously.

22. A method for inducing an immune response in an animal according to claims 16, 17, or 18 further comprising compounding said first and/or said second immunogenic component with an adjuvant.

23. A method for inducing an immune response in an animal according to claim 22 wherein said compounding is done using an adjuvant selected from the group consisting of aluminum salts, oil-in-water, saponin, cytokines, and oligonucleotides or polymeric molecules encoding immunostimulatory CpG motifs.

24. A method for inducing an immune response in an animal according to claim 23 wherein said compounding is done using aluminum salts selected from the group consisting of aluminum hydroxide, aluminum phosphate, and aluminum sulfate.

25. A method for inducing an immune response in an animal according to claim 23 wherein said compounding is done using oil-in-water adjuvants selected from the group consisting of MF59, SAF, Ribi™, complete Freunds adjuvant, and incomplete Freunds adjuvant.

26. A method for inducing an immune response in an animal according to claims 16, 17, or 18 wherein said native antigens are selected from the group consisting of polypeptides, proteins, polysaccharides, carbohydrates, nucleic acids and conjugates thereof.

27. A method for inducing an immune response in an animal according to claim 26 wherein said native antigens are derived from viruses, bacteria, fungi, or protozoans.

28. A method for inducing an immune response in an animal according to claims 16, 17, or 18 wherein said recombinant antigens are selected from the group consisting of polypeptides, proteins, polysaccharides, carbohydrates, nucleic acids and conjugates thereof.

29. A method for inducing an immune response in an animal according to claim 28 wherein said recombinant antigens are derived from viruses, bacteria, fungi, cancerous cells or protozoans.

30. A method for inducing an immune response in an animal according to claims 16, 17, or 18 wherein said immunogenic composition induces in its recipient an immune response that prevents, palliates or treats a disease.

31. A method for inducing an immune response in an animal according to claims 16, 17, or 18 wherein said immunogenic composition is a vaccine.

32. A method for inducing an immune response in an animal according to claim 31 wherein said animal is a human.

33. A method for inducing an immune response in an animal comprising:

priming an animal's immune system by administering an immunizing component selected from the group consisting of: native antigens, recombinant antigens, non-alphavirus viral vectors, nucleic acids, and alphavirus derived vector systems selected from the group consisting of alphavirus vector particles, eukaryotic layered vector initiation systems, and RNA vector replicons; and

boosting said animal's primed immune system by administering a second immunizing component selected from the group consisting of: native antigens, recombinant antigens, non-alphavirus viral vectors, nucleic acids, and alphavirus derived vector systems selected from the group consisting of alphavirus vector particles, eukaryotic layered vector initiation systems, and RNA vector replicons, with

the proviso that said first immunizing component is different from said second immunizing component and that said first immunizing component, said second immunizing component or both are alphavirus derived vector systems.

34. A method for inducing an immune response in an animal according to claim 33 wherein said immune response prevents, palliates or treats a disease.

35. A method for inducing an immune response in an animal according to claim 34 wherein said animal is a human.

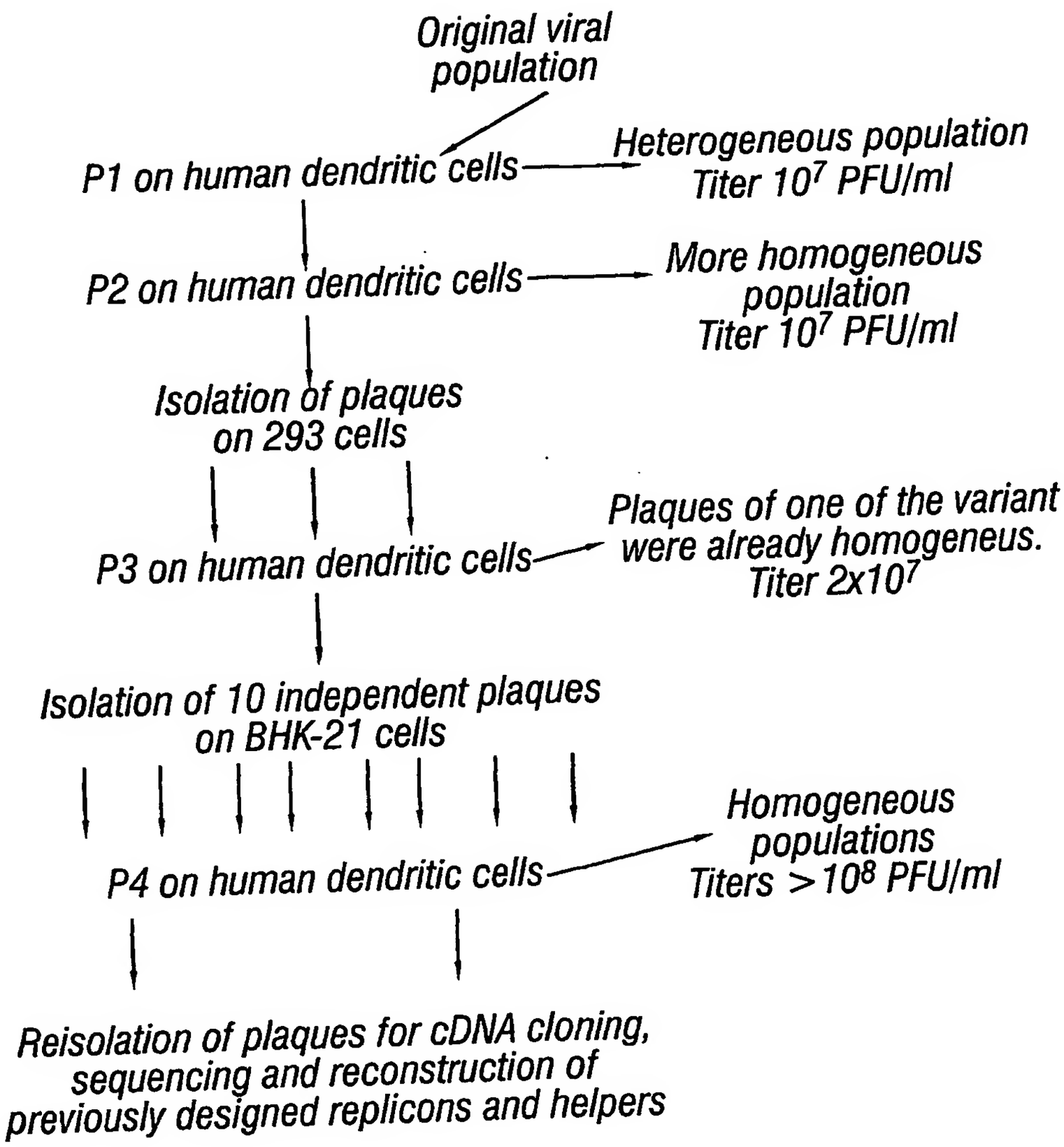
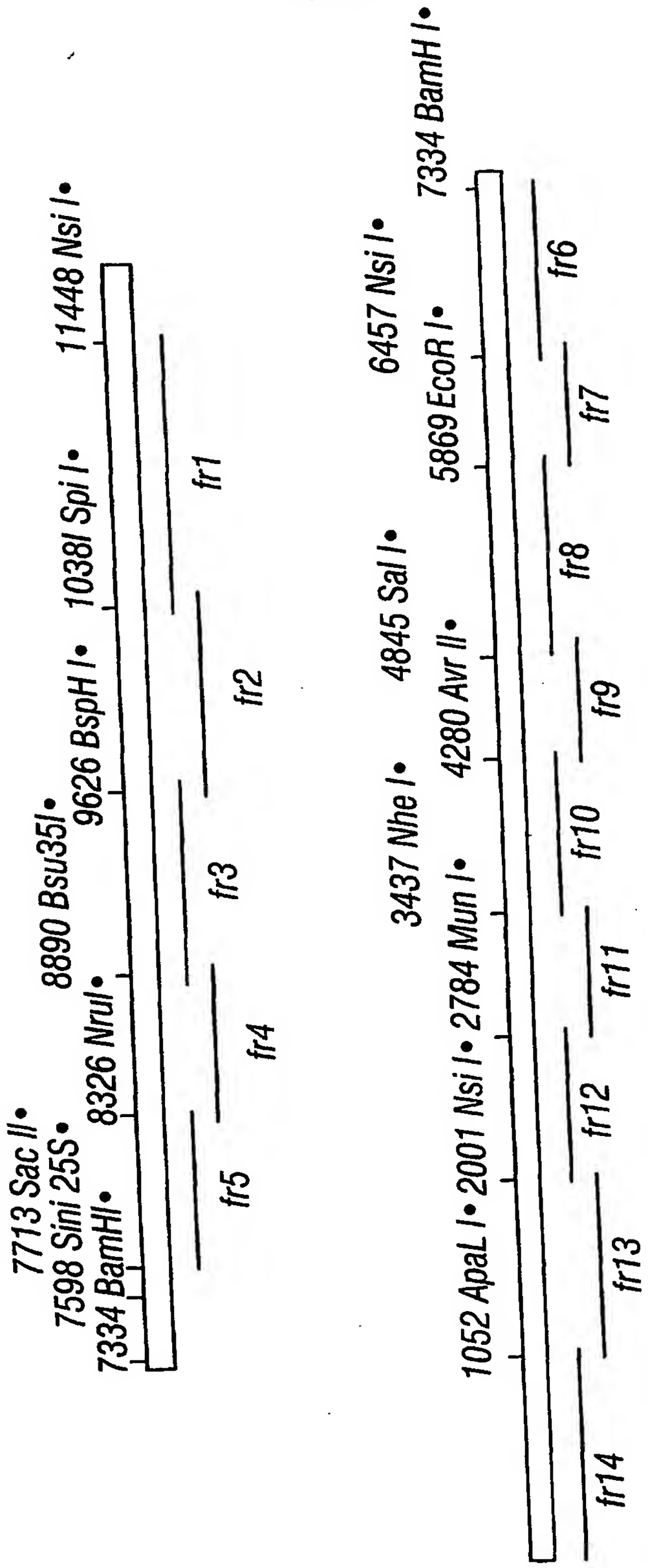

Original viral population
P1 on human dendritic cells
Heterogeneous population Titer 10^7 PFU/ml
P2 on human dendritic cells
More homogeneous population Titer 10^7 PFU/ml
Isolation of plaques on 293 cells
P3 on human dendritic cells
Plaques of one of the variant were already homogeneus. Titer 2x10^7
Isolation of 10 independent plaques on BHK-21 cells
P4 on human dendritic cells
Homogeneous populations Titers >10^8 PFU/ml
Reisolation of plaques for cDNA cloning, sequencing and reconstruction of previously designed replicons and helpers

FIG. 1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7598 Sini 25S•
7334 BamHI•
8326 NruI•
8890 Bsu35I•
9626 BspH I•
1038I Spi I•
1144 Nsi I•
fr5
fr4
fr3
fr2
fr1
1052 ApaL I• 2001 Nsi I• 2784 Mun I•
3437 Nhe I•
4845 Sal I•
4280 Avr II•
6457 Nsi I•
5869 EcoR I•
7334 BamH I•
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fr13

FIG. 2A

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FIG. 2B.1

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AGCAACCACCGAAGCCGAAGAAACCAAAAACGCAGGAGAAGAAGAAGAAGCAACCTGCAAAACCCAAACCCGGAA
AGAGACAGCGCATGGCACTTAAGTTGGAGGCCGACAGATCGTTCGACGTCAAGAACGAGGACGGAGATGTCATCG
GGCACGCACTGGCCATGGAAGGAAAGGTAATGAAACCTCTGCACGTGAAAGGAACCCCATCGACCACCCTGTGCTAT
CAAAGCTCAAATTTACCAAGTCGTCAGCATACGACATGGAGTTCGCACAGTTGCCAGTCAACATGAGAAGTGAGG

FIG. 2B.2

CATTCACCTACACCAGTGAACACCCCGAAGGATTCTATAACTGGCACCACGGAGCGGTGCAGTATAGTGGAGGTA
GATTTACCATCCCTCGCGGAGTAGGAGGCAGAGGAGACAGCGGTCGTCCGATCATGGATAACTCCGGTCgGGTTG
TCGCGATAGTCCTCGGTGGAGCTGATGAAGGAACACGAACTGCCCTTTCGGTCGTCACCTGGAATAGTAAAGGGA
AGACAATTAAGACGACCCCGGAAGGGACAGAAGAGTGGTCCGCAGCACCACTGGTCACGGAATGTGTTTGCTCG
GAAATGTGAGCTTCCCATGCGACCGCCCGCCCACATGCTATACCCGCGAACCTTCCAGAGCCCTCGACATCCTTG
AAGAGAACGTGAACCATGAGGCCTACGATACCCTGCTCAATGCCATATTGCGGTGCGGATCGTCTGGCAGAAGCA
AAAGAAGCGTCACTGACGACTTTACCCTGACCAGCCCCTACTTGGGCACATGCTCGTACTGCCATACTGAAC
CGTGCTTCAGCCCTGTTAAGATCGAGCAGGTCTGGGACGAAGCGGACGATAACACCATACGCATACAGACTTCCG
CCCAGTTTGGATACGACCAAAGCGGAGCAGCAAGCGCAAACAAGTACCGCTACATGTCGCTTAAGCAGGATCACA
CCGTTAAAGAAGGCACCATGGATGACATCAAGATTAGCACCTCAGGACCGTGTAGAAGGCTTAGCTACAAAGGAT
ACTTTCTCCTCGCAAAATGCCCTCCAGGGGACAGCGTAACGGTTAGCATAGTGAGTAGCAACTCAGCAACGTCAT
GTACACTGGCCCGCAAGATAAAACCAAAATTCGTGGGGACGGGAAAAATATGATCTACCTCCCGTTCACGGTAAAA
AAATTCCTTGCACAGTGTACGACCGTCTGAAAGGAACAACTGCAGGCTACATCACTATGCACAGGCCGGGACCGC
ACGCTTATACATCCTACCTGGAAGAATCATCAGGGAAAGTTTACGCAAAAGCCGCCATCTGGGAAGAACATTACGT
ATGAGTGCAAGTGCGGCGACTACAAGACCAGAACCGTTTCGACCCGCACCGAAATCACTGGTTGCACCGCCATCA
AGCAGTGCGTCGCCTATAAGAGCGACCAAACGAAGTGGGTCTTCAACTCACCGGACTTGATCAGACATGACGACC
ACACGGCCCAAGGGAAATTGCATTTGCCTTTCAAGTTGATCCCGAGTACCTGCATGGTCCCTGTTGCCCACGCGC
CGAATGTAATACATGGCTTTAAACACATCAGCCTCCAATTAGATACAGACCACTTGACATTGCTCACCACCAGGA
GACTAGGGGCAAACCCGGAACCAACCACTGAATGGATCGTCGGAAAGACGGTCAGAAACTTCACCGTCGACCGAG
ATGGCCTGGAATACATATGGGGAAATCATGAGCCAGTGAGGGTCTATGCCCAAGAGTCAGCACCAGGAGACCCTC
ACGGATGGCCACACGAAATAGTACAGCATTACTACCATCGCCATCCTGTGTACACCATCTTAGCCGTCGCATCAG
CTACCGTGGCGATGATGATTGGCGTAACTGTTGCAGTGTTATGTGCCTGTAAAGCGCGCCGTGAGTGCCTGACGC
CATACGCCCTGGCCCCAAACGCCGTAATCCCAACTTCGCTGGCACTCTTGTGCTGCGTTAGGTCGGCCAATGCTG
AAACGTTCACCGAGACCATGAGTTACTTGTGGTCGAACAGTCAGCCGTTCTTCTGGGTCCAGTTGTGCATACCTT
TGGCCGCTTTCATCGTTCTAATGCGCTGCTGCTCCTGCTCGCCTTTTTTAGTGGTTGCCGGCGCCTACCTGG
CGAAGGTAGACGCCTACGAACATGCGACCACTGTTCCAAATGTGCCACAGATACGGTATAAGGCACTTGTTGAAAA
GGGCAGGGTATGCCCCGCTCAATTTGGAGATCACTGTCATGTCCTCGGAGGTTTTGCCTTCCACCAACCAAGAGT
ACATTACCTGCAAATTCACCACTGTGGTCCCCTCCCCAAAAATCAAATGCTGCGGCTCCTTGGAATGTCAGCCGG
CCGTTCATGCAGACTATACCTGCAAGGTCTTCGGAGGGGTGTACCCCTTTATGTGGGGAGGAGCGCAATGTTTTT
GCGACAGTGAGAACAGCCAGATGAGTGAGGCGTACGTCGAACTGTCAGCAGATTGCGCGTCTGACCACGCGCAGG
CGATTAAGGTGCACACTGCCGCGATGAAAGTAGGACTGCGTATAGTGTACGGGAACACTACCAGTTTCCTAGATG
TGTACGTGAACGGAGTCACACCAGGAACGTCTAAAGACTTGAAAGTCATAGCTGGACCAATTTCAGCATCGTTTA
CGCCATTCGATCATAAGGTCGTTATCCATCGCGGCCTGGTGTACAACTATGACTTCCCGGAATATGGAGCGATGA
AACCAGGAGCGTTTGGACGACATTCAAGCTACCTCCTTGACTAGCAAGGATCTCATCGCCAGCACAGACATTAGGC
TACTCAAGCCTTCCGCCAAGAACGTGCATGTCCCGTACACGCAGGCCGCATCAGGATTTGAGATGTGGAAAAACA
ACTCAGGCCGCCCACTGCAGGAAACCGCACCTTTCGGGTGTAAGATTGCAGTAAATCCGCTCCGAGCGGTGGACT
GTTCATACGGGAACATTCCCATTTCTATTGACATCCCGAACGCTGCCTTTATCAGGACATCAGATGCACCACTGG
TCTCAACAGTCAAATGTGAAGTCAGTGAGTGCACTTATTCAGCAGACTTCGGCGGGATGGCCACCCTGCAGTATG
TATCCGACCGCGAAGGTCAATGCCCCGTACATTCGCATTCGAGCACAGCAACTCTCCAAGAGTCGACAGTACATG
TCCTGGAGAAAGGAGCGGTGACAGTACACTTTAGCACCGCGAGTCCACAGGCGAACTTTATCGTATCGCTGTGTG
GGAAGAAGACAACATGCAATGCAGAATGTAAACCACCAGCTGACCATATCGTGAGCACCCCGCACAAAAATGACC
AAGAATTTCAAGCCGCCATCTAAAAACATCATGGAGTTGGCTGTTGCCCTTTTCGGCGGCGCCTCGTCGCTAT
TAATTATAGGACTTATGATTTTTGCTTGCAGCATGATGTGACTAGCACACGAAGATGACCGCTACGCCCCAATG
ATCCGACCAGCAAAACTCGATGTACTTCCGAGGAACTGATGTGCATAATGCATcaggctggtacattagatccccgctta
ccgcgggcaatatagcaacactaaaaactcgatgtacttccgaggaagcgcagtgcataatgctgcgcagtgttgccaca
taaccactatattaaccatttatctagcggacgccaaaaactcaatgtatttctgaggaagcgtggtgcataatgccacg
cagcgtctgcataacttttattatttcttttattaatcaacaaaattttgtttttaacatttc

FIG. 2B.3

ATTGACGGCGTAGTACACACTATTGAATCAAACAGCCGACCAATTGCACTACCATCACAATGGAGAAGCCAGTAG
TAAACGTAGACGTAGACCCCAGAGTCCGTTTGTCGTGCAACTGCAAAAAAGCTTCCCGCAATTTGAGGTAGTAG
CACAGCAGGTCACTCCAAATGACCATGCTAATGCCAGAGCATTTTCGCATCTGGCCAGTAAACTAATCGAGCTGG
AGGTTCCTACCACAGCGACGATCTTGGACATAGGCAGCGCACCGGCTCGTAGAATGTTTTCCGAGCACCAGTATC
ATTGTGTCTGCCCCATGCGTAGTCCAGAAGACCCGGACCGCATGATGAAATATGCCAGTAAACTGGCGGAAAAAG
CGTGCAAGATTACAAACAAGAACTTGCATGAGAAGATTAAGGATCTCCGGACCGTACTTGATACGCCGGATGCTG
AAACACCATCGCTCTGCTTTCACAACGATGTTACCTGCAACATGCGTGCCGAATATTCCGTCATGCAGGACGTGT
ATATCAACGCTCCCGGAACTATCTATCATCAGGCTATGAAAGGCGTGCGGACCCTGTACTGGATTGGCTTCGACA
CCACCCAGTTCATGTTCTCGGCTATGGCAGGTTCGTACCCTGCGTACAACACCAACTGGGCCGACGAGAAAGTCC
TTGAAGCGCGTAACATCGGACTTTGCAGCACAAAGCTGAGTGAAGGTAGGACAGGAAAATTGTCGATAATGAGGA
AGAAGGAGTTGAAGCCCGGGTCGCGGGTTTATTTCTCCGTAGGATCGACACTTTATCCAGAACACAGAGCCAGCT
TGCAGAGCTGGCATCTTCCATCGGTGTTCCACTTGAATGGAAAGCAGTCGTACACTTGCCGCTGTGATACAGTGG
TGAGTTGCGAAGGCTACGTAGTGAAGAAAATCACCATCAGTCCCGGGATCACGGGAGAAACCGTGGGATACGCGG
TTACACACAATAGCGAGGGCTTCTTGCTATGCAAAGTTACTGACACAGTAAAAGGAGAACGGGTATCGTTCCCTG
TGTGCACGTACATCCCGGCCACCATATGCGATCAGATGACTGGTATAATGGCCACGGATATATCACCTGACGATG
CACAAAAACTTCTGGTTGGGCTCAACCAGCGAATTGTCATTAACGGTAGGACTAACAGGAACACCAACACCATGC
AAAATTACCTTCTGCCGATCATAGCACAAGGGTTCAGCAAATGGGCTAAGGAGCGCAAGGATGATCTTGATAACG
AGAAAATGCTGGGTACTAGAGAACGCAAGCTTACGTATGGCTGCTTGTGGGCGTTTCGCACTAAGAAAGTACATT
CGTTTTATCGCCCACCTGGAACGCAGACCATCGTAAAAGTCCCAGCCTCTTTTAGCGCTTTTCCCATGTCGTCCG
TATGGACGACCTCTTTGCCCATGTCGCTGAGGCAGAAATTGAAACTGGCATTGCAACCAAAGAAGGAGGAAAAAC
TGCTGCAGGTCTCGGAGGAATTAGTCATGGAGGCCAAGGCTGCTTTTGAGGATGCTCAGGAGGAAGCCAGAGCGG
AGAAGCTCCGAGAAGCACTTCCACCATTAGTGGCAGACAAAGGCATCGAGGCAGCCGCAGAAGTTGTCTCTGCGAAG
TGGAGGGGCTCCAGGCGGACATCGGAGCAGCATTAGTTGAAACCCCGCGCGGTCACGTAAGGATAATACCTCAAG
CAAATGACCGTATGATCGGACAGTATATCGTTGTCTCGCCAAACTCTGTGCTGAAGAATGCCAAACTCGCACCAG
CGCACCCGCTAGCAGATCAGGTTAAGATCATAACACACTCCGGAAGATCAGGAAGGTACGCGGTCGAACCATACG
ACGCTAAAGTACTGATGCCAGCAGGAGGTGCCGTACCATGGCCAGAATTCCTAGCACTGAGTGAGAGCGCCACGT
TAGTGTACAACGAAAGAGAGTTTGTGAACCGCAAACTATACCACATTGCCATGCATGGCCCCGCCAAGAATACAG
AAGAGGAGCAGTACAAGGTTACAAAGGCAGAGCTTGCAGAAACAGAGTACGTGTTTGACGTGGACAAGAAGCGTT
GCGTTAAGAAGGAAGAAGCCTCAGGTCTGGTCCTCTCGGGAGAACTGACCAACCCTCCTATCATGAGCTAGCTC
TGGAGGGACTGAAGACCCGACCTGCGGTCCCGTACAAGGTCGAAACAATAGGAGTGATAGGCACACCGGGGTCGG
GCAAGTCAGCTATTATCAAGTCAACTGTCACGGCACGAGATCTTGTTACCAGCGGAAAGAAAATTGTCGCG
AAATTGAGGCCGACGTGCTAAGACTGAGGGGTATGCAGATTACGTCGAAGACAGTAGATTCGGTTATGCTCAACG
GATGCCACAAAGCCGTAGAAGTGCTGTACGTTGACGAAGCGTTCGCGTGCCACGCAGGAGCACTACTTGCCTGA
TTGCTATCGTCAGGCCCCGCAAGAAGGTAGTACTATGCGGAGACCCCATGCAATGCGGATTCTTCAACATGATGC
AACTAAAGGTACATTTCAATCACCCTGAAAAAGACATATGCACCAAGACATTCTACAAGTATATCTCCCGGCGTT
GCACACAGCCAGTTACAGCTATTGTATCGACACTGCATTACGATGGAAAGATGAAAACCACGAACCCGTGCAAGA
AGAACATTGAAATCGATATTACAGGGGCCACAAAGCCGAAGCCAGGGGATATCATCCTGACATGTTTCCGCGGGT
GGGTTAAGCAATTGCAAATCGACTATCCCGGACATGAAGTAATGACAGCCGCGGCCTCACAAGGGCTAACCAGAA
AAGGAGTGTATGCCGTCCGGCAAAAAGTCAATGAAAACCCACTGTACGCGATCACATCAGAGCATGTGAACGTGT
TGCTCACCCGCACTGAGGACAGGCTAGTGTGGAAAACCTTGCAGGGCGACCCATGGATTAAGCAGCTCACTAACA
TACCTAAAGGAAACTTTCAGGCTACTATAGAGGACTGGGAAGCTGAACACAAGGGAATAATTGCTGCAATAAACA
GCCCCACTCCCCGTGCCAATCCGTTCAGCTGCAAGACCAACGTTTGCTGGGCGAAAGCATTGGAACCGATACTAG
CCACGGCCGGTATCGTACTTACCGGTTGCCAGTGGAGCGAACTGTTCCCACAGTTTGCGGATGACAAACCACATT
CGGCCATTTACGCCTTAGACGTAATTTGCATTAAGTTTTTCGGCATGGACTTGACAAGCGGACTGTTTTCTAAAC
AGAGCATCCCACTAACGTACCATCCCGCCGATTCAGCGAGGCCGGTAGCTCATTGGGACAACAGCCCAGGAACCC
GCAAGTATGGGTACGATCACGCCATTGCCGCCGAACTCTCCCGTAGATTTCCGGTGTTCCAGCTAGCTGGGAAGG
GCACACAACTTGATTTGCAGACGGGGAGAACCAGAGTTATCTCTGCACAGCATAACCTGGTCCCGGTGAACCGCA
ATCTTCCTCACGCCTTAGTCCCCGAGTACAAGGAGAAGCAACCCGGCCCGGTCGAAAAATTCTTGAACCAGTTCA
AACACCACTCAGTACTTGTGGTATCAGAGGAAAAAATTGAAGCTCCCCGTAAGAGAATCGAATGGATCGCCCCGA
TTGGCATAGCCGGTGCAGATAAGAACTACAACCTGGCTTTCGGGTTTCCGCCGCAGGCACGGTACGACCTGGTGT
TCATCAACATTGGAACTAAATACAGAAACCACCACTTTCAGCAGTGCGAAGACCATGCGGCGACCTTAAAAACCC
TTTCGCGTTCGGCCCTGAATTGCCTTAACCCAGGAGGCACCCTCGTGGTGAAGTCCTATGGCTACGCCGACCGCA
ACAGTGAGGACGTAGTCACCGCTCTTGCCAGAAAGTTTGTCAGGGTGTCTGCAGCGAGACCAGATTGTGTCTCAA
GCAATACAGAAATGTACCTGATTTTCCGACAACTAGACAACAGCCGTACACGGCAATTCACCCCGCACCATCTGA
ATTGCGTGATTTCGTCCGTGTATGAGGGTACAAGAGATGGAGTTGGAGCCGCGCCGTCATACCGCACCAAAAGGG
AGAATATTGCTGACTGTCAAGAGGAAGCAGTTGTCAACGCAGCCAATCCGCTGGGTAGACCAGGCGAAGGAGTCT
GCCGTGCCATCTATAAACGTTGGCCGACCAGTTTTACCGATTCAGCCACGGAGACAGGCACCGCAAGAATGACTG
TGTGCCTAGGAAAGAAAGTGATCCACGCGGTCGGCCCTGATTTCCGGAAGCACCCAGAAGCAGAAGCCTTGAAAT
TGCTACAAAACGCCTACCATGCAGTGGCAGACTTAGTAAATGAACATAACATCAAGTCTGTCGCCATTCCACTGC
TATCTACAGGCATTTACGCAGCCGGAAAAGACCGCCTTGAAGTATCACTTAACTGCTTGACAACCGCGCTAGACA

FIG. 2C.1

GAACTGACGCGGACGTAACCATCTATTGCCTGGATAAGAAGTGGAAGGAAATCGACGCGGCACTCCAACTTA
AGGAGTCTGTAACAGAGCTGAAGGATGAAGATATGGAGATCGACGATGAGTTAGTATGGATCCATCCAGACAGTT
GCTTGAAGGGAAGAAAGGGATTCAGTACTACAAAAGGAAAATTGTATTCGTACTTCGAAGGCACCAAATTCCATC
AAGCAGCAAAAGACATGGCGGAGATAAAGGTCCTGTTCCCTAATGACCAGGAAAGTAATGAACAACTGTGTGCCT
ACATATTGGGTGAGACCATGGAAGCAATCCGCGAAAAGTGCCCGGTCGACCATAACCCGTCGTCTAGCCCGCCCA
AAACGTTGCCGTGCCTTTGCATGTATGCCATGACGCCAGAAAGGGTCCACAGACTTAGAAGCAATAACGTCAAAG
AAGTTACAGTATGCTCCTCCACCCCCCTTCCTAAGCACAAAATTAAGAATGTTCAGAAGGTTCAGTGCACGAAAG
TAGTCCTGTTTAATCCGCACACTCCCGCATTCGTTCCCGCCCGTAAGTACATAGAAGTGCCAGAACAGCCTACCG
CTCCTCCTGCACAGGCCGAGGAGGCCCCCGAAGTTGTAGCGACACCGTCACCATCTACAGCTGATAACACCTCGC
TTGATGTCACAGACATCTCACTGGATATGGATGACAGTAGCGAAGGCTCACTTTTTTCGAGCTTTAGCGGATCGG
ACAACTCTATTACTAGTATGGACAGTTGGTCGTCAGGACCTAGTTCACTAGAGATAGTAGACCGAAGGCAGGTGG
TGGTGGCTGACGTTCATGCCGTCCAAGAGCCTGCCCCTATTCCACCGCCAAGGCTAAAGAAGATGGCCCGCCTGG
CAGCGGCAAGAAAAGAGCCCACTCCACCGGCAAGCAATAGCTCTGAGTCCCTCCACCTCTCTTTTGGTGGGGTAT
CCATGTCCCTCGGATCAATTTTCGACGGAGAGACGGCCCGCCAGGCAGCGTACAACCCCTGGCAACAGGCCCCA
CGGATGTGCCTATGTCTTTCGGATCGTTTTCCGACGGAGAGATTGATGAGCTGAGCCGCAGAGTAACTGAGTCCG
AACCCGTCCTGTTTGGATCATTTGAACCGGGCGAAGTGAACTCAATTATATCGTCCCGATCAGCCGTATCTTTTC
CACTACGCAAGCAGAGACGTAGACGCAGGACGAGGAGGACTGAATACTGACTAACCGGGGTAGGTGGGTACATAT
TTTCGACGGACACAGGCCCTGGGCACTTGCAAAAGAAGTCCGTTCTGCAGAACCAGCTTACAGAACCGACCTTGG
AGCGCAATGTCCTGGAAAGAATTCATGCCCCGGTGCTCGACACGTCGAAAGAGGAACAACTCAAACTCAGGTACC
AGATGATGCCCACCGAAGCCAACAAAAGTAGGTACCAGTCTCGTAAAGTAGAAAATCAGAAAGCCATAACCACTG
AGCGACTACTGTCAGGACTACGACTGTATAACTCTGCCACAGATCAGCCAGAATGCTATAAGATCACCTATCCGA
AACCATTGTACTCCAGTAGCGTACCGGCGAACTACTCCGATCCACAGTTCGCTGTAGCTGTCTGTAACAACTATC
TGCATGAGAACTATCCGACAGTAGCATCTTATCAGATTACTGACGAGTACGATGCTTACTTGGATATGGTAGACG
GGACAGTCGCCTGCCTGGATACTGCAACCTTCTGCCCCGCTAAGCTTAGAAGTTACCCGAAAAAACATGAGTATA
GAGCCCCGAATATCCGCAGTGCGGTTCCATCAGCGATGCAGAACACGCTACAAAATGTGCTCATTGCCGCAACTA
AAAGAAATTGCAACGTCACGCAGATGCGTGAACTGCCAACACTGGACTCAGCGACATTCAATGTCGAATGCTTTC
GAAAATATGCATGTAATGACGAGTATTGGGAGGAGTTCGCTCGGAAGCCAATTAGGATTACCACTGAGTTTGTCA
CCGCATATGTAGCTAGACTGAAAGGCCCTAAGGCCGCCGCACTATTTGCAAAGACGTATAATTTGGTCCCATTGC
AAGAAGTGCCTATGGATAGATTCGTCATGGACATGAAAAGGGACGTGAAAGTTACACCAGGCACGAAACACACAG
AAGAAAGACCGAAAGTACAAGTGATACAAGCCGCAGAACCCCTGGCGACTGCTTACTTATGCGGATTCACCGGG
AATTAGTGCGTAGGCTTACGGCCGTCTTGCTTCCAAACATTCACACCGCTTTTTGACATGTCGGCGGAGGATTTTG
ATGCAATCATAGCAGAACACTTCAAGCAAGGCGACCCGGTACTGGAGACGGATATCGCATCATTCGACAAAAGCC
AAGACGACGCTATGGCGTTAACCGGTGTGATGATCTTGGAGGACCTGGGTGTGGATCAACCACTACTCGACTTGA
TCGAGTGCGCCTTTGGAGAAATATCATCCACCCATCTACCTACGGGTACTCGTTTTAAATTCGGGGCGATGATGA
AATCCGGAATGTTCCTCACACTTTTTGTCAACACAGTTTTGAATGTCGTTATCGCCAGCAGAGTACTAGAAGAGC
GGCTTAAAACGTCCAGATGTCGAGCAGCGTTCATTGGCGACAACATCATACATGGAGTAGTATCTGACAAAGAAA
TGGCTGAGAGGTGCGCCACCTGGCTCAACATGGAGGTTAAAGTCATCGACGCAGTCTCGGTGAGACCACCTT
ACTTCTGCGGCGGATTTATCTTGCAAGATTCGGTTACTTCCACAGCGTGCCGCGTGGCGGACCCctgaaaaggc
tgtttaagttgggtaaaccgctcccagccgacgacgagcaagacgaagacagaagacgcgctctgctagatgaaa
caaaggcgtggtttagagtaggtataacaggcactttagcagtggccgtgacgacccggtatgaggtagacaata
ttacacctgtcctactggcattgagaacttttgcccagagcaaaagagcattccaagccatcagaggggaaataa
agcatctctacggtggtcctaaatagtcagcatagtacatttcatctgactaatactacaacaccaccaccatga
atagaggattctttaacatgctcggccgcgccgcccttcccggccccactgccatgtggaggccgcggAGAAGGA
GGCAGGCGGCCCCGATGCCTGCCCGCAACGGGCTGGCTTCTCAAATCCAGCAACTGACCACAGCCGTCAGTGCCC
TAGTCATTGGACAGGCAACTAGACCTCAACCCCCACGTCCACGCCCGCCACCGCGCCAGAAGAAGCAGGCGCCCA
AGCAACCACCGAAGCCGAAGAAACCAAAAACGCAGGAGAAGAAGAAGAAGCAACCTGCAAAACCCAAACCCGGAA
AGAGACAGCGCATGGCACTTAAGTTGGAGGCCGACAGATCGTTCGACGTCAAGAACGAGGACGGAGATGTCATCG
GGCACGCACTGGCCATGGAAGGAAAGGTAATGAAACCTCTGCACGTGAAAGGAACCCCATCGACCACCCTGTGCTAT
CAAAGCTCAAATTTACCAAGTCGTCAGCATACGACATGGAGTTCGCACAGTTGCCAGTCAACATGAGAAGTGAGG
CATTCACCTACACCAGTGAACACCCCGAAGGATTCTATAACTGGCACCACGGAGCGGTGCAGTATAGTGGAGGTA
GATTTACCATCCCTCGCGGAGTAGGAGGCAGAGGAGACAGCGGTCGTCCGATCATGGATAACTCCGGTCgGGTTG
TCGCGATAGTCCTCGGTGGAGCTGATGAAGGAACACGAACTGCCCTTTCGGTCGTCACCTGGAATAGTAAAGGGA
AGACAATTAAGACGACCCCGGAAGGGACAGAAGAGTGGTCCGCAGCACCACTGGTCACGGAATGTGTTTGCTCG
GAAATGTGAGCTTCCCATGCGACCGCCCGCCCACATGCTATACCCGCGAACCTTCCAGAGCCCTCGACATCCTTG
AAGAGAACGTGAACCATGAGGCCTACGATACCCTGCTCAATGCCATATTGCGGTGCGGATCGTCTGGCAGAAGCA
AAAGAAGCGTCACTGACGACTTTACCCTGACCAGCCCCTACTTGGGCACATGCTCGTACTGCCATACTGAAC
CGTGCTTCAGCCCTGTTAAGATCGAGCAGGTCTGGGACGAAGCGGACGATAACACCATACGCATACAGACTTCCG
CCCAGTTTGGATACGACCAAAGCGGAGCAGCAAGCGCAAACAAGTACCGCTACATGTCGCTTAAGCAGGATCACA
CCGTTAAAGAAGGCACCATGGATGACATCAAGATTAGCACCTCAGGACCGTGTAGAAGGCTTAGCTACAAAGGAT
ACTTTCTCCTCGCAAAATGCCCTCCAGGGGACAGCGTAACGGTTAGCATAGTGAGTAGCAACTCAGCAACGTCAT

FIG. 2C.2

GTACACTGGCCCGCAAGATAAAACCAAAATTCGTGGGGACGGGAAAAATATGATCTACCTCCCGTTCACGGTAAAA
AAATTCCTTGCACAGTGTACGACCGTCTGAAAGGAACAACTGCAGGCTACATCACTATGCACAGGCCGGGACCGC
ACGCTTATACATCCTACCTGGAAGAATCATCAGGGAAAGTTTACGCAAAAGCCGCCATCTGGGAAGAACATTACGT
ATGAGTGCAAGTGCGGCGACTACAAGACCAGAACCGTTTCGACCCGCACCGAAATCACTGGTTGCACCGCCATCA
AGCAGTGCGTCGCCTATAAGAGCGACCAAACGAAGTGGGTCTTCAACTCACCGGACTTGATCAGACATGACGACC
ACACGGCCCAAGGGAAATTGCATTTGCCTTTCAAGTTGATCCCGAGTACCTGCATGGTCCCTGTTGCCCACGCGC
CGAATGTAATACATGGCTTTAAACACATCAGCCTCCAATTAGATACAGACCACTTGACATTGCTCACCACCAGGA
GACTAGGGGCAAACCCGGAACCAACCACTGAATGGATCGTCGGAAAGACGGTCAGAAACTTCACCGTCGACCGAG
ATGGCCTGGAATACATATGGGGAAATCATGAGCCAGTGAGGGTCTATGCCCAAGAGTCAGCACCAGGAGACCCTC
ACGGATGGCCACACGAAATAGTACAGCATTACTACCATCGCCATCCTGTGTACACCATCTTAGCCGTCGCATCAG
CTACCGTGGCGATGATGATTGGCGTAACTGTTGCAGTGTTATGTGCCTGTAAAGCGCGCCGTGAGTGCCTGACGC
CATACGCCCTGGCCCCAAACGCCGTAATCCCAACTTCGCTGGCACTCTTGTGCTGCGTTAGGTCGGCCAATGCTG
AAACGTTCACCGAGACCATGAGTTACTTGTGGTCGAACAGTCAGCCGTTCTTCTGGGTCCAGTTGTGCATACCTT
TGGCCGCTTTCATCGTTCTAATGCGCTGCTGCTCCTGCTCGCCTTTTTTAGTGGTTGCCGGCGCCTACCTGG
CGAAGGTAGACGCCTACGAACATGCGACCACTGTTCCAAATGTGCCACAGATACCGTATAAGGCACTTGTTGAAA
GGGCAGGGTATGCCCCGCTCAATTTGGAGATCACTGTCATGTCCTCGGAGGTTTTGCCTTCCACCAACCAAGAGT
ACATTACCTGCAAATTCACCACTGTGGTCCCCTCCCCAAAAATCAAATGCTGCGGCTCCTTGGAATGTCAGCCGG
CCGTTCATGCAGACTATACCTGCAAGGTCTTCGGAGGGGTGTACCCCTTTATGTGGGGAGGAGCGCAATGTTTTT
GCGACAGTGAGAACAGCCAGATGAGTGAGGCGTACGTCGAACTGTCAGCAGATTGCGCGTCTGACCACGCGCAGG
CGATTAAGGTGCACACTGCCGCGATGAAAGTAGGACTGCGTATAGTGTACGGGAACACTACCAGTTTCCTAGATG
TGTACGTGAACGGAGTCACACCAGGAACGTCTAAAGACTTGAAAGTCATAGCTGGACCAATTTCAGCATCGTTTA
CGCCATTCGATCATAAGGTCGTTATCCATCGCGGCCTGGTGTACAACTATGACTTCCCGGAATATGGAGCGATGA
AACCAGGAGCGTTTGGACGACATTCAAGCTACCTCCTTGACTAGCAAGGATCTCATCGCCAGCACAGACATTAGGC
TACTCAAGCCTTCCGCCAAGAACGTGCATGTCCCGTACACGCAGGCCGCATCAGGATTTGAGATGTGGAAAAACA
ACTCAGGCCGCCCACTGCAGGAAACCGCACCTTTCGGGTGTAAGATTGCAGTAAATCCGCTCCGAGCGGTGGACT
GTTCATACGGGAACATTCCCATTTCTATTGACATCCCGAACGCTGCCTTTATCAGGACATCAGATGCACCACTGG
TCTCAACAGTCAAATGTGAAGTCAGTGAGTGCACTTATTCAGCAGACTTCGGCGGGATGGCCACCCTGCAGTATG
TATCCGACCGCGAAGGTCAATGCCCCGTACATTCGCATTCGAGCACAGCAACTCTCCAAGAGTCGACAGTACATG
TCCTGGAGAAAGGAGCGGTGACAGTACACTTTAGCACCGCGAGTCCACAGGCGAACTTTATCGTATCGCTGTGTG
GGAAGAAGACAACATGCAATGCAGAATGTAAACCACCAGCTGACCATATCGTGAGCACCCCGCACAAAAATGACC
AAGAATTTCAAGCCGCCATCTAAAAACATCATGGAGTTGGCTGTTGCCCTTTTCGGCGGCGCCTCGTCGCTAT
TAATTATAGGACTTATGATTTTTGCTTGCAGCATGATGTGACTAGCACACGAAGATGACCGCTACGCCCCAATG
ATCCGACCAGCAAAACTCGATGTACTTCCGAGGAACTGATGTGCATAATGCATcaggctggtacattagatcccc
gcttaccgcgggcaatatagcaacactaaaaactcgatgtacttccgaggaagcgcagtgcataatgctgcgcag
tgttgccacataaccactatattaaccatttatctagcggacgccaaaaactcaatgtatttctgaggaagcgtg
gtgcataatgccacgcagcgtctgcataacttttattatttcttttattaatcaacaaaattttgtttttaacat
ttc

FIG. 2C.3

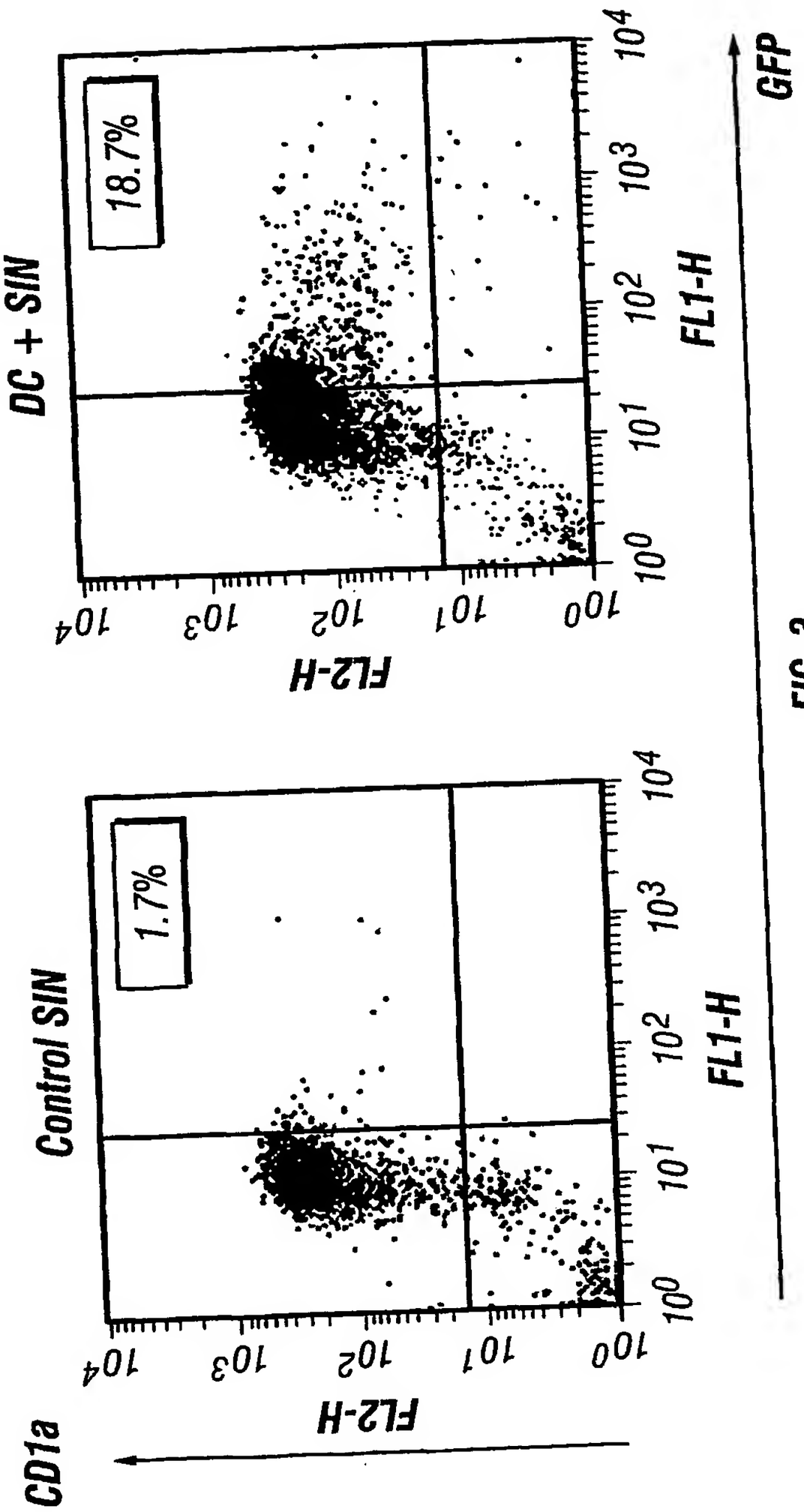


FIG. 3

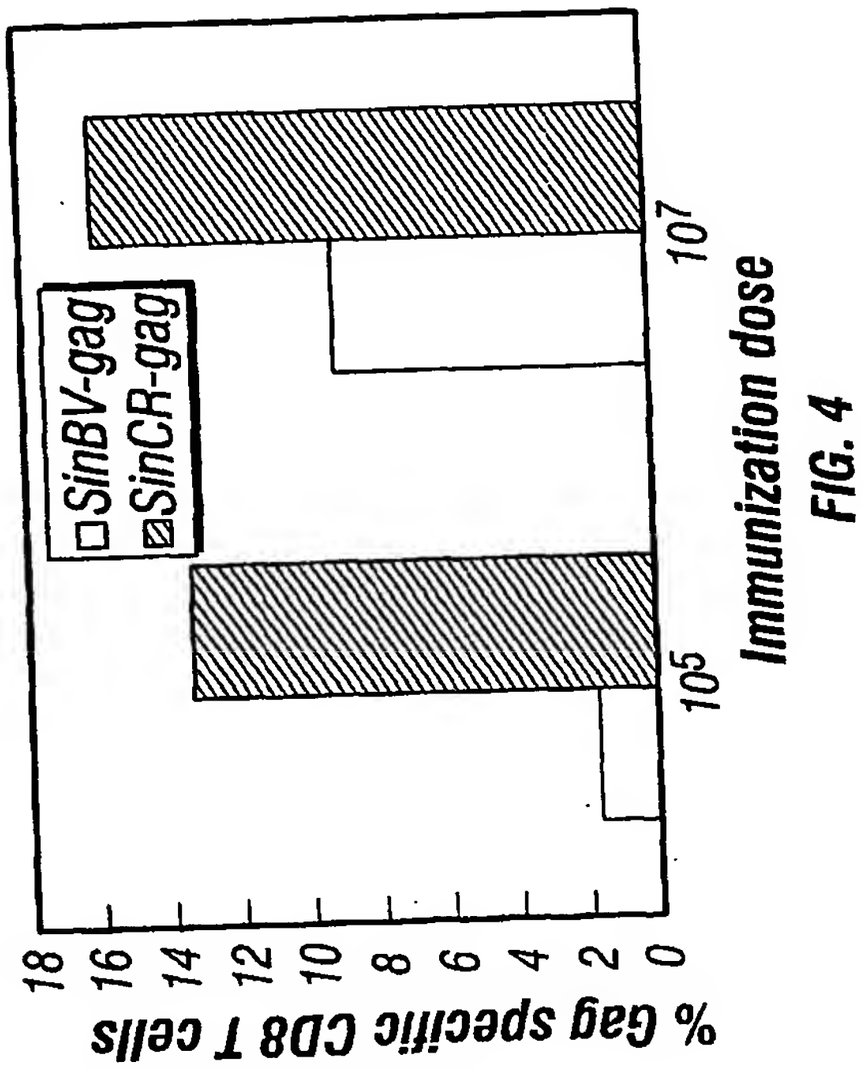

% Gag specific CD8 T cells
18
16
14
12
10
8
6
4
2
0
SinBV-gag
SinCR-gag
10^5
10^7
Immunization dose

FIG. 4

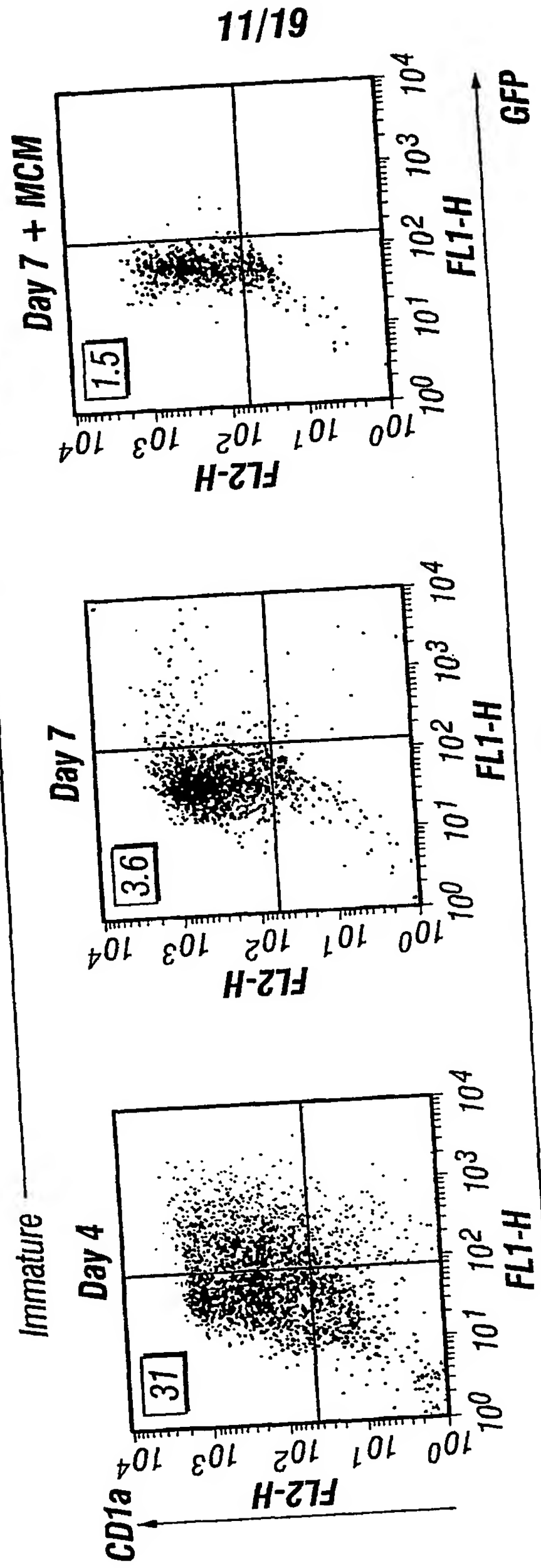

Mature
Day 7 + MCM
1.5
FL2-H
FL1-H
GFP
Day 7
3.6
FL2-H
FL1-H
Immature
Day 4
CD1a
31
FL2-H
FL1-H

FIG. 5

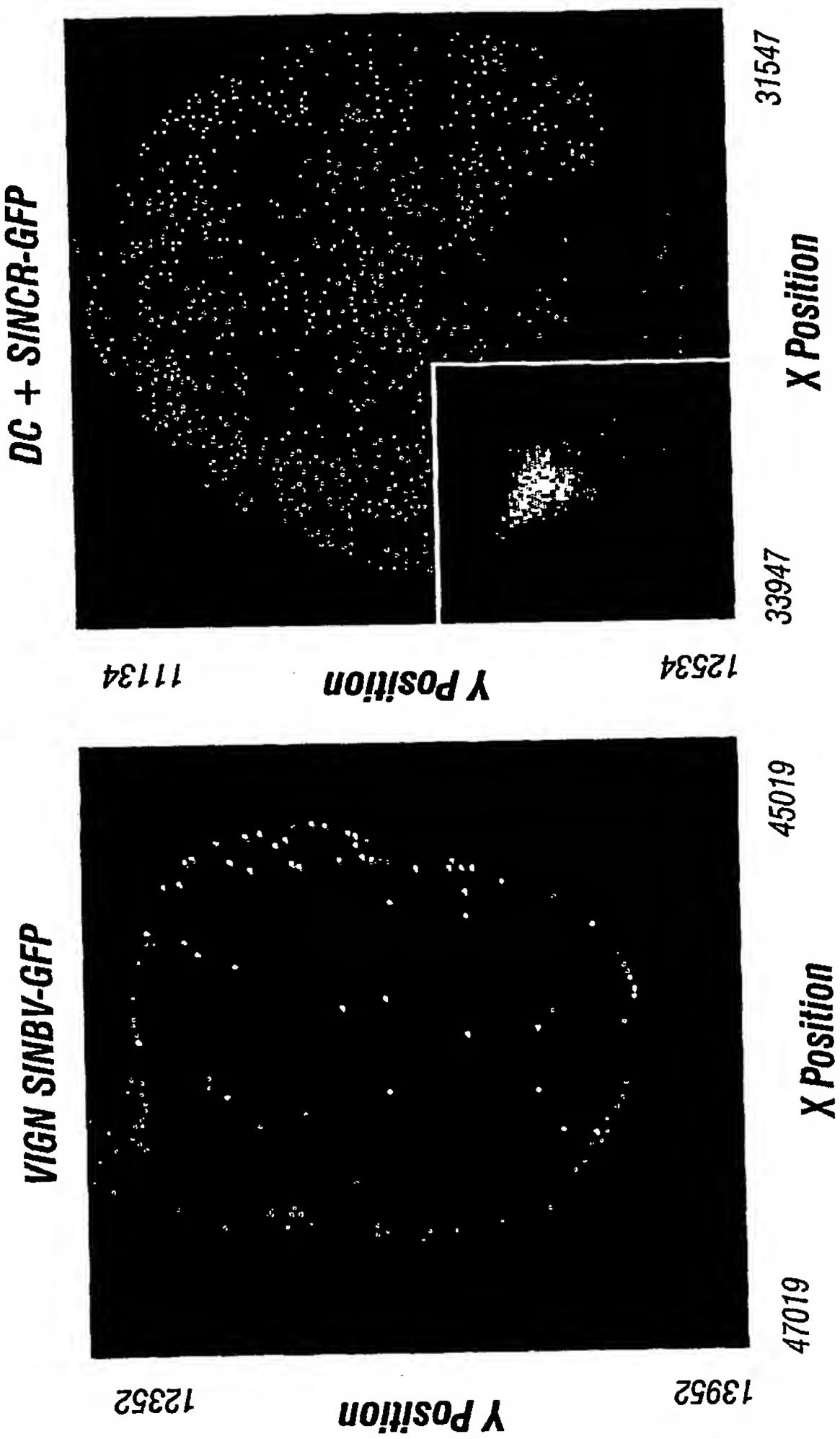
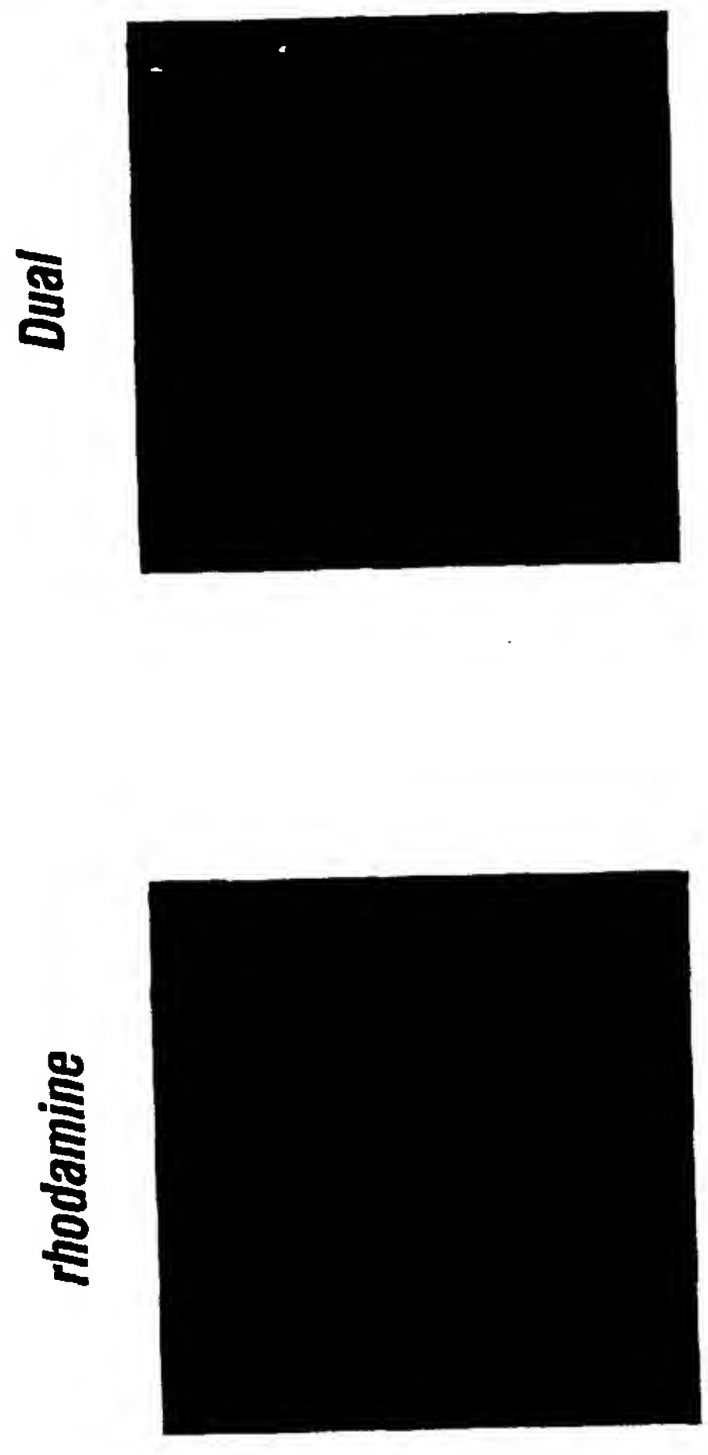

DC + SINCR-GFP
11134
Y Position
12534
31547
33947
X Position
VIGN SINBV-GFP
12352
Y Position
13952
45019
47019
X Position

FIG. 6



X20

FIG. 7

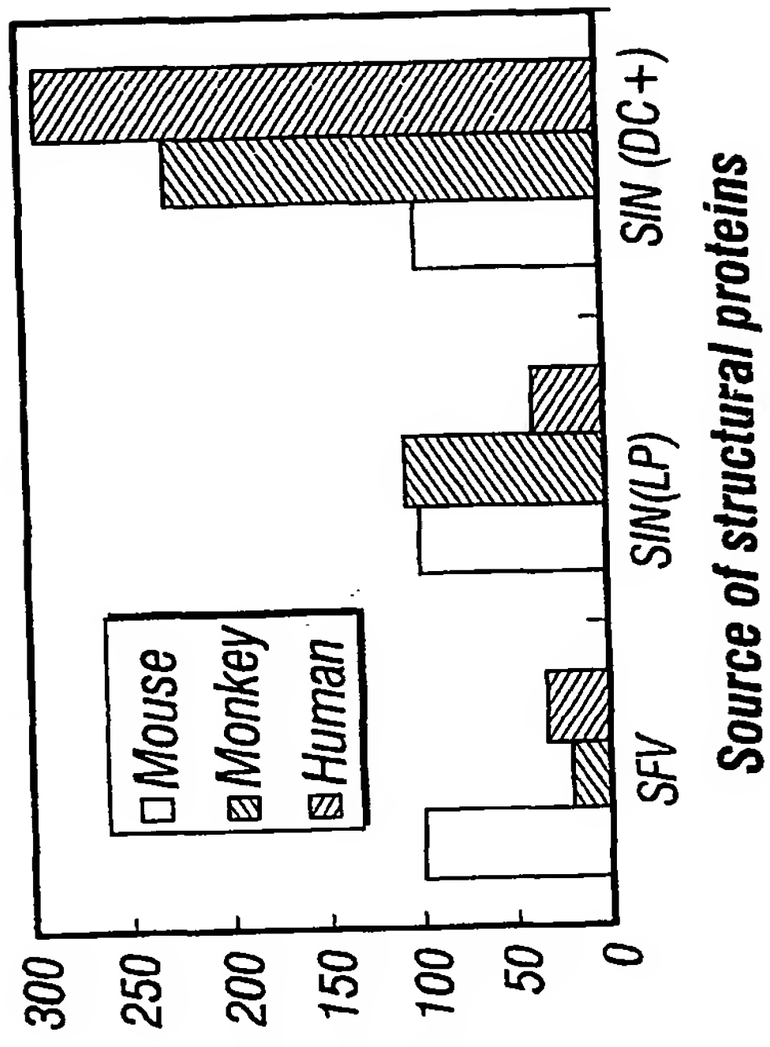

% Mouse DC transduction efficiency
300
250
200
150
100
50
0
Mouse
Monkey
Human
SFV
SIN(LP)
SIN (DC+)
Source of structural proteins

FIG. 8

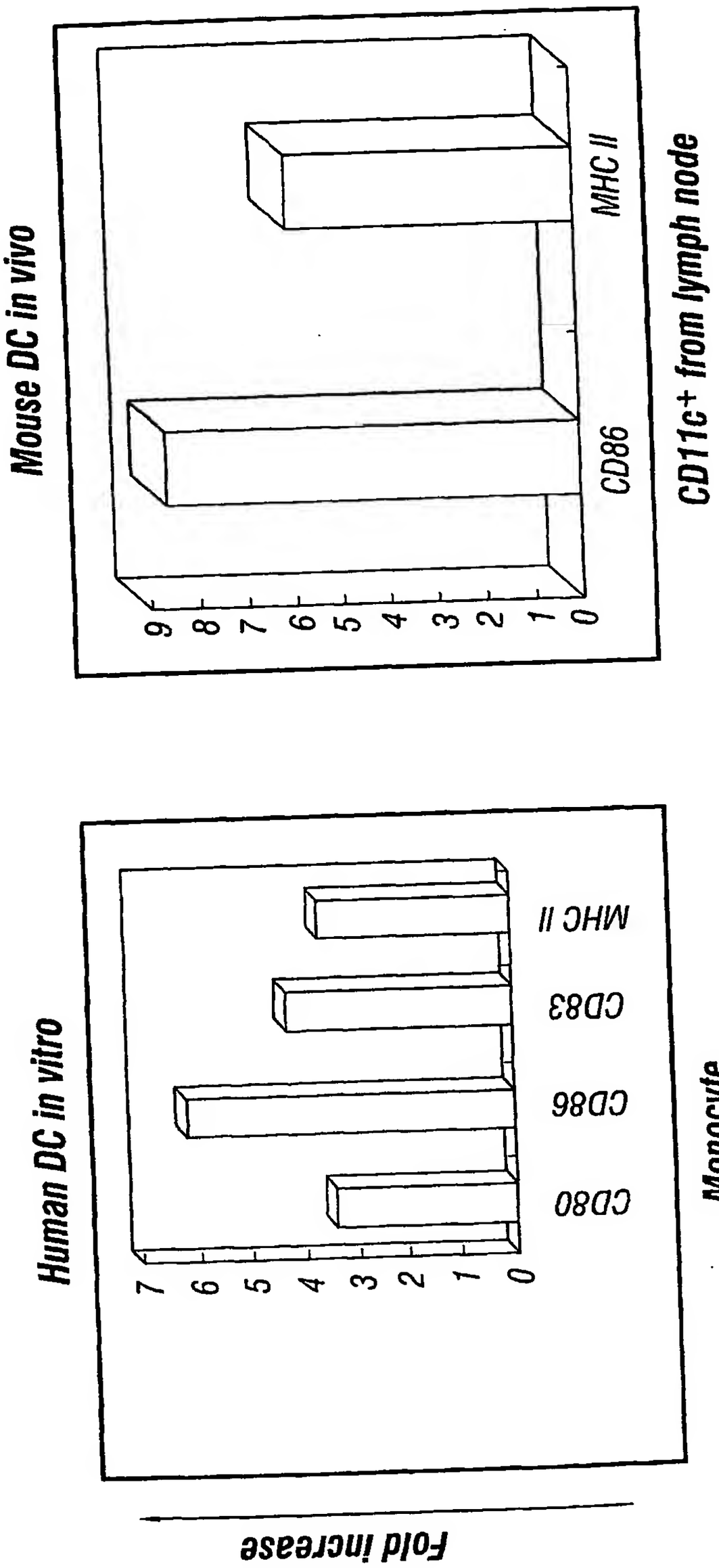

Mouse DC in vivo
CD11c+ from lymph node
MHC II
CD86
0
1
2
3
4
5
6
7
8
9
Human DC in vitro
Monocyte
MHC II
CD83
CD86
CD80
0
1
2
3
4
5
6
7
Fold increase

FIG. 9

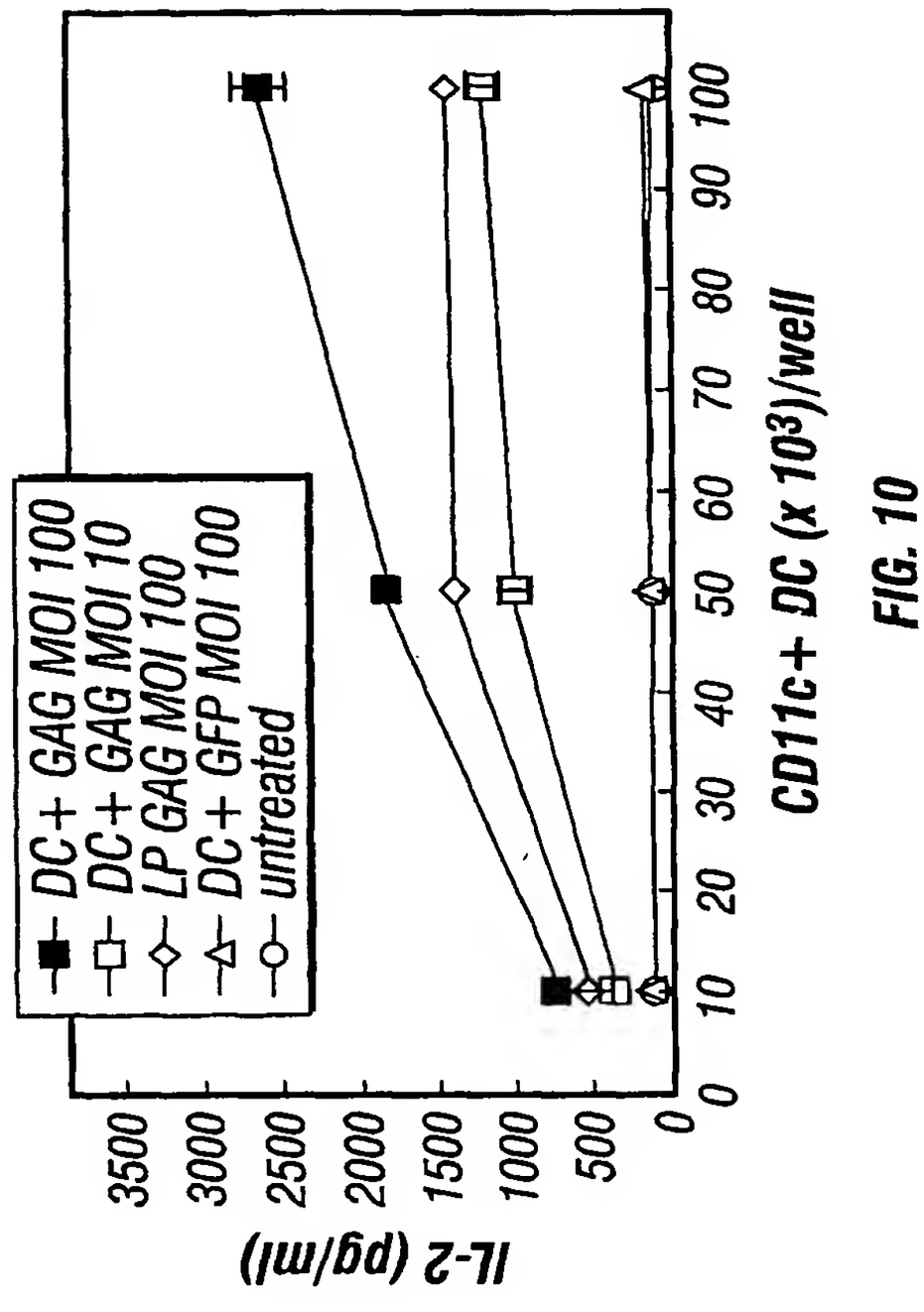

IL-2 (pg/ml)
3500
3000
2500
2000
1500
1000
500
0
0
10
20
30
40
50
60
70
80
90
100
CD11c+ DC (x 10³)/well
DC+ GAG MOI 100
DC+ GAG MOI 10
LP GAG MOI 100
DC+ GFP MOI 100
untreated

FIG. 10

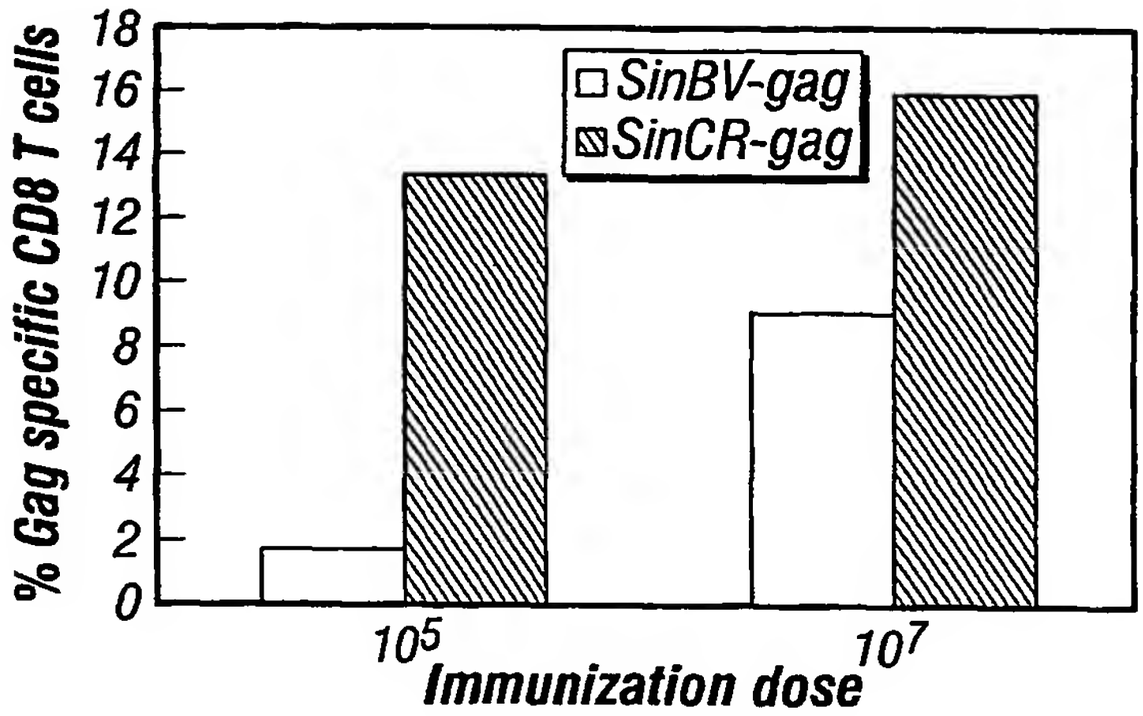

% Gag specific CD8 T cells
18
16
14
12
10
8
6
4
2
0
SinBV-gag
SinCR-gag
10^5
10^7
Immunization dose

FIG. 11

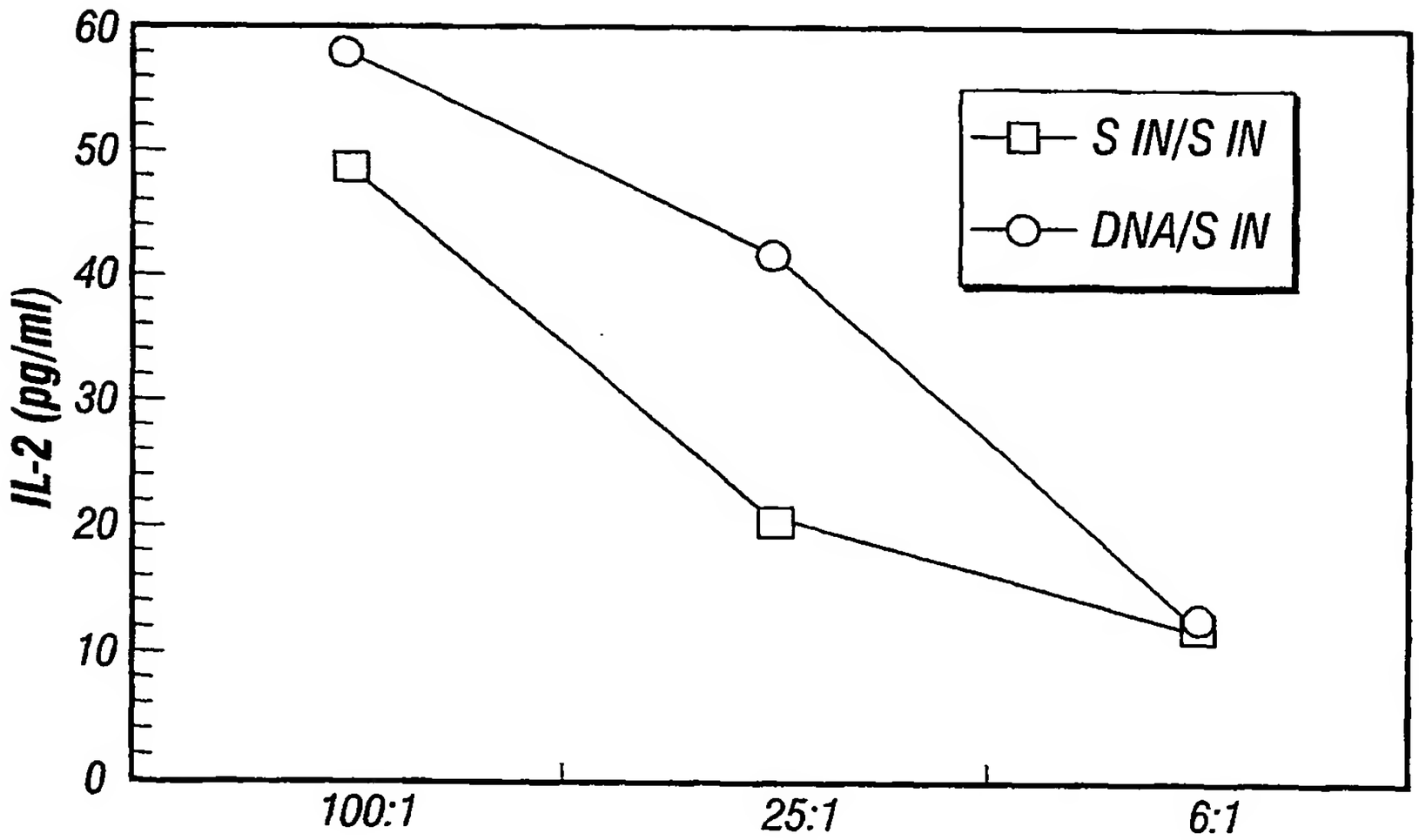

IL-2 (pg/ml)
60
50
40
30
20
10
0
S IN/S IN
DNA/S IN
100:1
25:1
6:1

FIG. 12

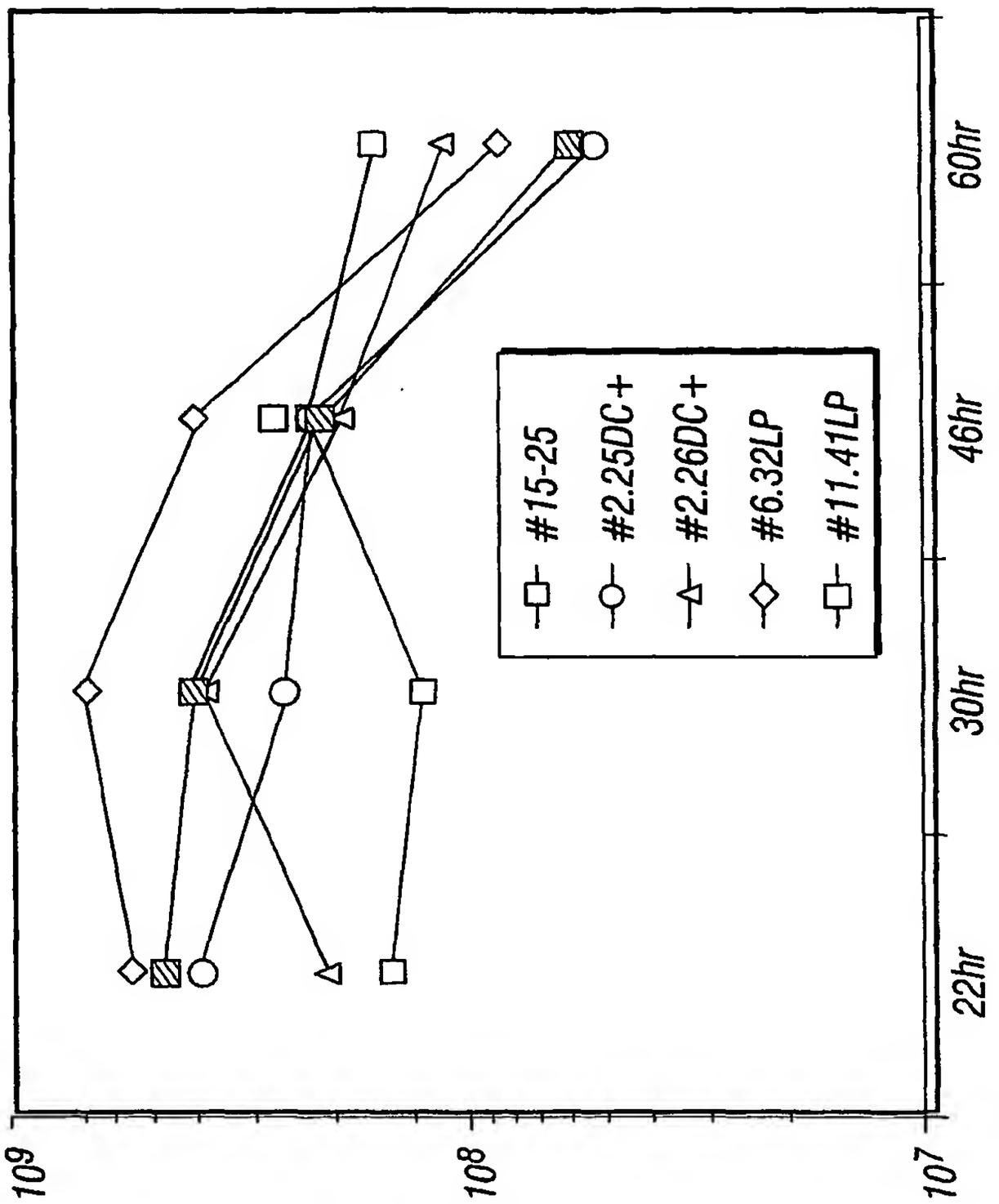

Crude particle titer (IU/mL)
10^9
10^8
10^7
22hr
30hr
46hr
60hr
Complete media exchange
#15-25
#2.25DC+
#2.26DC+
#6.32LP
#11.41LP

FIG. 13

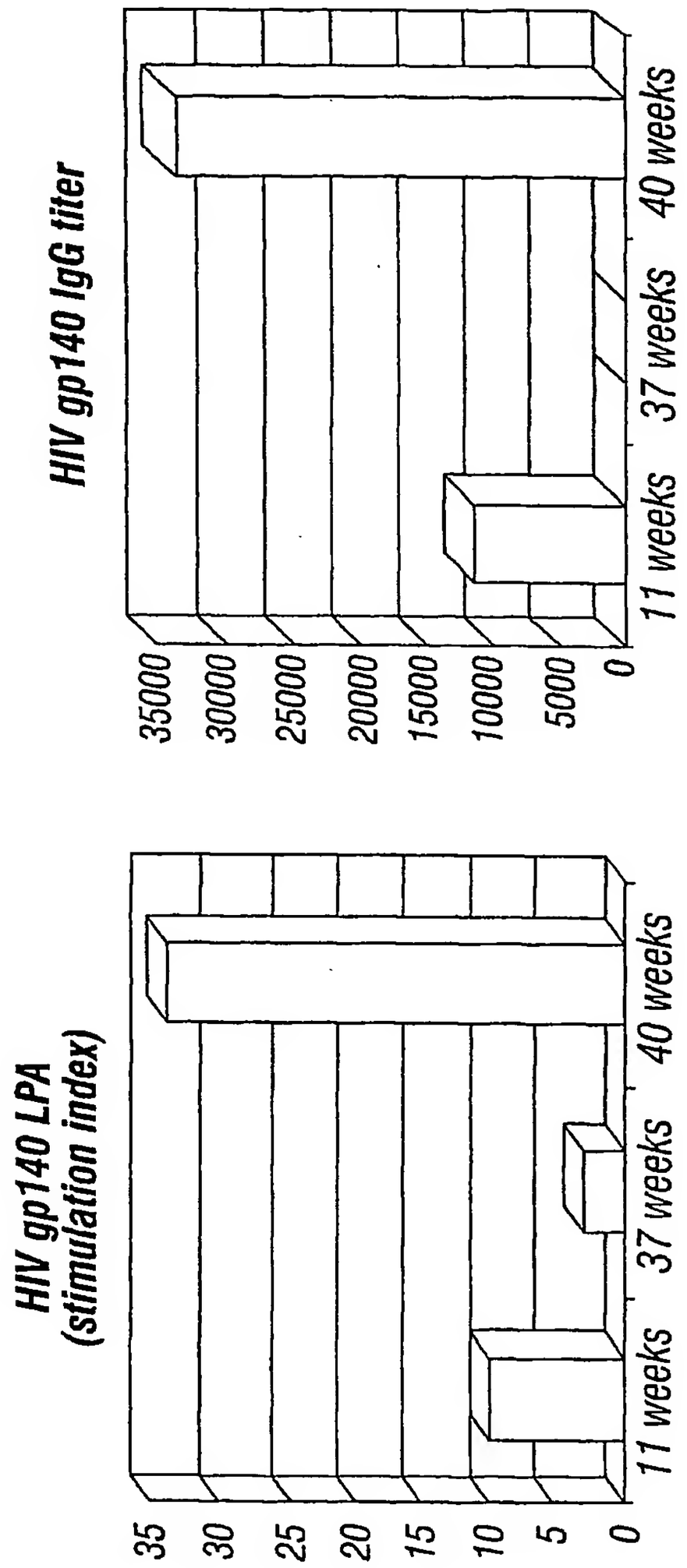

HIV gp140 IgG titer
35000
30000
25000
20000
15000
10000
5000
0
11 weeks
37 weeks
40 weeks
HIV gp140 LPA
(stimulation index)
35
30
25
20
15
10
5
0
11 weeks
37 weeks
40 weeks

FIG. 14